QUANTIFYING FITNESS COSTS IN A VECTOR-BORNE

PARASITE SYSTEM: EXPERIMENTS WITH

PIGEONS, HIPPOBOSCID FLIES, AND

AN AVIAN MALARIA PARASITE

by

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STATEMENT OF DISSERTATION APPROVAL

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ABSTRACT

The host, the parasite, and the vector each shape disease dynamics. Vector-borne parasites spread by (1) getting into the next vertebrate host from an infected vector, and (2) getting into the next vector from an infected vertebrate host. I use an experimental approach to investigate pairwise interactions between organisms in a system composed of a vertebrate host, the Rock Pigeon *(Columba livia)*, a hippoboscid fly vector *(Pseudolynchia canariensis)*, and a malaria parasite of the pigeon *(Haemoproteus columbae)*. Ultimately, such studies may reveal how ecological interactions shape evolutionary processes.

Transmission requires an infected vector bite a vertebrate host. Fewer parasites would be transmitted if hosts could defend themselves against vectors. I tested the effectiveness of anti-vector defense by manipulating two pigeon defenses against flies: preening and antibody responses. Each independently decreased fly longevity and the defenses work additively. However, they were ineffective in decreasing malaria parasite transmission. This ineffectiveness may have little immediate consequence for the pigeon. In a field experiment *H. columbae* had no effect on nestling pigeon growth, survival, or fledging success. This was surprising since *H. columbae* is correlated with lower survival in older pigeons; however, nestling pigeons are provided a particularly rich diet by both parents and may be tolerant to infection.

To complete transmission, the vector must bite an infected vertebrate host, but the effect of the infected blood on the arthropod host is unknown. I found malaria parasites decrease fly survival and fecundity, but only for female flies. Both sexes feed on blood and transmit parasites, but the comparatively high female reproductive costs may decrease infection tolerance through energetic constraints. Females also take larger meals to fuel reproduction, which may increase their exposure to parasites.

In my work I found malaria harms arthropod hosts more than vertebrate hosts, counter to the conventional wisdom that a parasite should not harm its vector. However, if a "vector" is defined by host mobility, pigeons may be the actual vectors in this system compared to the more sedentary flies. Disease dynamics here may also differ because both fly sexes transmit the parasite. These two points warrant further investigation.

I dedicate this dissertation to Carolyn Raiford, the high school
science teacher who inspired me to
become a biologist.

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in my field. Lastly, I thank the birds and flies that gave their lives to science.

CHAPTER 1

INTRODUCTION

The ecology of parasites drives their evolutionary trajectory, and can predict local

abundance, transmission, and epidemiology. However, for many parasites vectored by

arthropods, very little is known about how the parasites affect their vector, or how vectors

interact with a vertebrate host. Often interest in disease symptoms of the host that are

caused by the parasite overshadows these equally important interactions, and limits much

of the research effort to the host-parasite interface. However, for arthropod-borne

parasites, vertebrate hosts encounter parasites only by interacting with the arthropod, and

so the nature and outcome of this interaction is key to understanding parasite

transmission. Additionally, for many parasites, including all of the malaria parasites, the

sexual stage of the parasite life cycle takes place in the arthropod, making the arthropod

the definitive host rather than the vertebrate. In such cases, the interaction between a

parasite and its arthropod host takes on additional importance for understanding the

evolutionary ecology of the parasite. My research attempts to understand the nature of

such understudied interactions using an experimental approach in a natural host-vector-

parasite system consisting of feral Rock Pigeons *(Columba livia)*, a pigeon malaria

parasite *(Haemoproteus columbae)*, and the hippoboscid fly vector *(Pseudolynchia*

canariensis). One goal of attempting to delineate the fitness impacts on each organism

involved in such interactions is to predict the value and/or importance of such

interactions in other systems. The ultimate goal of such work is to generally improve our understanding of the transmission dynamics, ecology, and evolution of vector-borne parasites.

1.1 Why All of the Interactions in Vector-Borne Disease Systems Are Important

Often studies on arthropod-borne parasites focus only on the symptoms of disease that a parasite causes in a particular host, with further research working towards treating disease symptoms. The majority of research on infectious disease, perhaps not surprisingly, has been on humans or other animals or plants that are important for agricultural, commercial, or (more rarely) for conservation purposes.

Much research has focused only on the interaction between parasite and vertebrate host (such as for vaccine or drug development), yet this interaction is just one of many interactions in a vector-borne disease system. Parasites also interact with their arthropod host, which likely plays an equal role in shaping parasite evolution and is critical to transmission ecology. Additionally, the interaction between vertebrate and arthropod host has been relatively understudied; instead, the focus has been on preventing this interaction from occurring. By broadening the scope of host-vector-parasite research to include these additional types of interactions, the better we can fully understand the ecology and evolution of arthropod-borne parasites, and possibly determine novel strategies to prevent transmission. Indeed, these are the stated goals of the United States research agencies on disease (NIAID Malaria Working Group 2008 booklet and CDC webpage for malaria research); but progress has been slow, particularly for non-human malaria parasites. Nonhuman work may progress faster as there are fewer ethical

restrictions to consider. Thoroughly examining and understanding multiple non-human malaria parasite systems will more broadly inform our understanding of disease ecology and potentially provide novel ideas for the control and, ultimately, eradication of malaria parasite species and other vector-borne pathogens.

1.2 Why Use a Study System of Pigeons, Flies, and Malaria Parasites

For reasons which defy analysis, louse-flies are particularly repellent insects, and most people experience a shudder of disgust at the sight of them, and are filled with a quite unreasonable feeling of horror if they happen to dart up their sleeves or into their hair while handling the host.
-Rothschild and Clay (1952) Fleas, Flukes, and Cuckoos Pg. 213

The system in which I chose to study host, vector, and parasite interactions is neither glamorous nor for the faint of heart as Rothschild and Clay (1952) describe. However, feral Rock Pigeons *(Columba livia)*, pigeon louse-flies *(*Hippoboscoidea: *Pseudolynchia canariensis)*, and the pigeon malaria parasite *Haemoproteus columbae* constitute a uniquely powerful natural system because of the relative ease with which all organisms can be maintained and experimentally manipulated in captivity.

Additionally, there is a long history of the study of all of these organisms, likely due to the ease with which pigeons can be maintained in captivity, e.g., (Adie 1915, Coatney 1931, Lastra and Coatney 1950, Ahmed and Mohammed 1978a, 1978b). Indeed, the life cycle of *H. columbae* has been studied since the late 1800s (Celli and Sanfelice 1891) and *P. canariensis* was the known vector of this parasite as early as 1906 (Sergent and Sergent 1906). *H. columbae* has also had a role in the study of human malaria parasites; determining the complete life cycle of human *Plasmodium* parasites was largely

advanced by critical studies of *H. columbae* in 1897 followed by studies of other bird malarias as model systems (Cox 2010).

The feral pigeons used in the following experiments were trapped from populations in the Salt Lake City, UT area. In Salt Lake City, prevalence of *H. columbae* is approximately 66%, with some seasonal variation (Appendix A). Where manipulations of immune response to the vector were central to the hypotheses tested, pigeons that had never been exposed to the bites of hippoboscid flies were required. These pigeons were bred in captivity from wild-caught parents in a fly-free facility, and the resulting F1 birds were used in experiments.

The hippoboscid flies that infest pigeons are patchily distributed among pigeon populations in the Salt Lake City valley (Harbison et al. 2008). The prevalence of flies has been shown to be correlated with the prevalence of the malaria parasite they transmit, *H. columbae* (Sol et al. 2000). The flies spend the majority of their time on pigeons (about 90%, *pers. obs.*), leaving only when disturbed by the bird, or, in the case of the female fly, to deposit offspring in the surrounding substrate. Female flies have an energetically expensive reproductive cycle: eggs eclose inside the female uterus and proceed through all three larval stages while still inside the female (Harwood and James 1979). The larva feed on milk glands within the female, and a single larva is deposited at a time (Coatney 1931). Male flies will often mate guard and will follow the female flies off the bird during these times; males can frequently be found guarding females when off of the host, presumably to prevent other males from mating (Coatney 1931, Yuval 2006). Both male and female flies feed approximately twice daily for 20-80 minutes at a time (Arcoverde et al. 2009).

When the flies take an infected blood meal, male and female *H. columbae* parasites in the sexual gametocyte stages are taken up with the meal. Once inside the fly, male parasites exflagellate, dividing into multiple motile parasites, and move to fertilize the female gametocytes in the fly gut. A very brief zygote stage forms, which quickly turns into an oocyst that develops on the outside of the midgut wall (Adie 1924). The oocyst, when mature, bursts and releases parasites in the sporozoite stage which migrate to the salivary glands where they can be injected into a pigeon when the fly takes another blood meal (Adie 1915). It takes 10-12 days for *H. columbae* to reach the sporozoite stage in the salivary glands after their eruption of oocysts in the midgut. It is the sporozoite stage that is transmissible to a pigeon.

Once in a pigeon host, the sporozoites migrate to the lung epithelial tissue where they replicate asexually, releasing small parasites into the peripheral red blood cells of the bird that then grow to the mature gametocyte stages infective to the fly. While in the bird blood parasites consume resources from the blood cell, including glucose (Manwell and Loeffler 1961). If more than two parasites invade the same blood cell, that cell will burst. Anemia has been reported in feral pigeons with especially heavy *H. columbae* infections (Markus and Oosthuizen 1972), and disease symptoms and pathology have been noted in especially heavily infected birds (Earle et al. 1993). However, chronic effects of *H. columbae* are more commonly found in free-ranging pigeons (Sol et al. 2003).

Natural systems of host, vector, and parasite are hard to study in the field and can be difficult to manipulate in captivity. Often manipulations are unethical in systems that involve human hosts. The development of new "model" natural systems is very much needed. A unique advantage of the pigeon-fly-malaria parasite system is that it provides

the opportunity to test whether female and male flies are differentially affected by the malaria parasite, and thus also provides a unique window into their comparative ecology (Chapter 5). Previous work on nonhuman malarias has focused on domesticated bird species, such as chickens and canaries where natural vectors are unknown or combinations of bird and parasite do not naturally occur (Bennett et al. 1993). A notable exception in a natural malaria parasite system are the studies of the lizard malaria parasite *Plasmodium mexicanum* by the Schall group, e.g., Vardo-Zalik and Schall (2009) and Schall (2011). Careful work on additional natural systems will allow for comparative studies and greater generalization of findings.

1.3 General Methods and Means of Experimentation

The experimental approaches described in this dissertation vary depending on the hypotheses being tested, and methods specific to particular experiments are described in the appropriate chapters. However, there are some general methods that were common across experiments that were beyond the scope of the individual chapters/publications, but nonetheless may prove useful for future researchers for rearing and maintaining birds and flies, and for manipulating the malaria parasite infection status of each. To provide a better understanding of the system and capabilities for experimentation for the reader and for future workers, these techniques are described here.

1.3.1 Maintaining and Breeding Rock Pigeons *(Columba livia)*

Rock Pigeons were caught using walk-in traps baited with a mix of grain formulated for pigeons in Salt Lake City, Utah, USA. Trapping locations included interstate overpasses along I-15 (3300 S, 4500 S, 5300 S, 7200 S, 12300 S), and permission from building owners at Pollyanna apartments (F street and 2nd Ave), Artspace Center (500 W

200 S), and the University of Utah campus (257 S 1400 E). Following capture, pigeons were transported to the University of Utah animal facility, where they were individually housed in wire mesh cages (30 x 30 x 56 cm). Birds were given *ad libitum* food, water and grit and kept on a 12-hour light/dark cycle. The majority of experiments used Rock Pigeons trapped in this way. However, while *H. columbae* infection status of trapped pigeons could be determined by repeated blood sampling (see Chapter 5 for methods) or PCR (Appendix B), a pigeon's history of exposure to the vector of this parasite *(P. canariensis)* was unknown even if its blood appeared uninfected. The exposure history of pigeons to flies was critical for the experiments described in Chapter 3 where the role of anti-fly immunity in vector defense and malaria parasite transmission was tested. To generate pigeons that had never been exposed to either flies or malaria parasites, we bred pigeons in captivity in a fly-free facility.

Pigeons chose a mate prior to breeding. Mate choice was determined by releasing two to six pigeons into an enclosed space and pairs that were observed to courtship fed and/or copulated were assumed to have selected their mate. Pigeons were bred in captivity either as mated pairs in a breeding cage (45 x 45 x 52 cm) with nest trays, or in an open room with several pairs freely flying with rectangular nest boxes provided, divided into two square sections large enough for pigeons to brood nestlings in each. Pigeons often have "double clutches" where they are sitting on eggs of the second clutch while still providing supplementary food to offspring from their first clutch (Johnston and Janiga 1995). In the open room setting, parentage was tracked by observing the identity of pairs mating and brooding or feeding nestlings (both parents contribute to these activities). In both cases and pine needles to served as nesting material. Pigeons successfully produced offspring

in both scenarios; young that could feed independently were removed then from the cages of their parents (at about 30 - 35 days of age). Pigeon F1 offspring were at least 6 months of age before being used in any experiments; after 6 months pigeons are sexually mature.

1.3.2 Maintaining and Breeding Hippoboscid Flies
(Pseudolynchia canariensis)

Originally, flies were collected from infested pigeons that were brought into captivity. Descriptions of how populations of flies were maintained on a single pigeon kept in cages surrounded by netting can be found in Chapter 5; additional details are provided here. Flies fed and bred in culture, and females deposited puparia between or under the newspaper layers in the pigeon cages. On average, puparia take 25 - 30 days to eclose under the temperature and humidity conditions in the animal rooms (20 - 25°C, 50-70% rH). Time to eclosion was observed with freshly deposited puparia that were collected and held at constant temperature and 50-70% rH in incubators. Puparium eclosion is temperature dependent, with warmer temperatures speeding eclosion (Figure 1.1).

Puparia were collected with forceps by searching the collection trays below cages. They were collected every two weeks to prevent eclosion between collection times and to maintain a clean environment for the birds. All puparia that were collected were brought to an incubator where the flies were allowed to eclose in glass vials with foam stoppers at 27 °C and approximately 60% rH.

H. columbae is not transmitted transovarially from the female fly to her offspring, so all flies eclosed in the incubator are were uninfected. This created a source of uninfected flies for experiments, or for adding back into the fly culture to maintain numbers. Adult
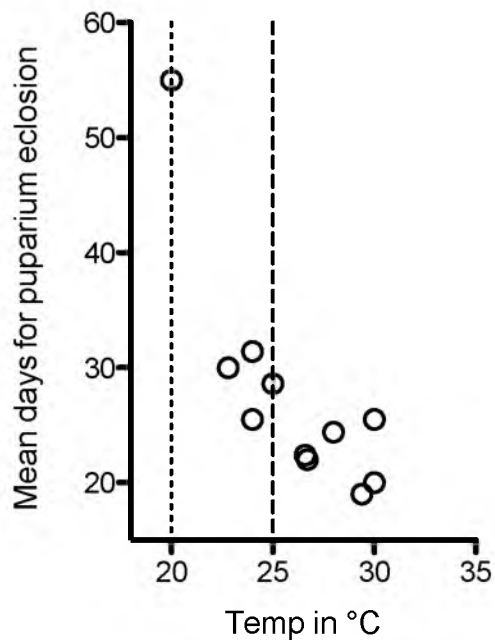
Figure 1.1. The number of days that *P. canariensis* puparia take to eclose varies with temperature. After 37 °C most puparia will not eclose (only 1.2% (Klei 1971)) as it is too hot, and below 13 °C puparia will not eclose because it is too cold. Animal rooms where populations of flies were kept on pigeons for either experiments or in culture were kept at a minimum temperature of 20 °C (dotted line), and a maximum temperature of 25 °C (dashed line). Temperature and eclosion data for 25 and 28 °C were provided by Kari Smith *(unpublished data)* and the rest of the points can be found in Sergent and Sergent (1906), Herath (1966), and Klei (1971).

fly longevity is approximately 5-7 weeks on average, dependent on host immunity, preening behavior, and temperature, among other factors. It takes about 6 days for female flies to feed, reach sexual maturity, mate, and produce their first offspring (Herath 1966, Klei 1971). Once mature, females produce an average of 2-3 puparia per week (an average of one offspring every 2 - 3 days at peak reproduction) for their lifetime.

1.3.3 Blood Sampling For Parasite Detection and ELISAs

Blood was taken from pigeons by brachial venipuncture using 27-gauge needles. Blood collected for the purpose of parasite detection was taken from the puncture site with a heparinized capillary tube. Blood dots were then put into frosted-end glass microscope slides and thin blood smears were made (see Appendix A). Additional dots were put into circles of Whatman 110 (4" diameter) filter paper for preservation for later molecular analyses. Layers of clean filter paper then separated the filter paper layers that contained blood dots. Filter papers were put into sandwich-size Ziploc bags, and smaller bags placed into larger Ziploc freezer bags, and stored at -20 °C for later DNA extraction with a Qiagen DNeasy blood and tissue kit (according to the established protocol for the kit) and PCR or Quantitative PCR analysis. PCR was used for *H. columbae* detection, and Quantitative PCR was used for examining ratios of *H. columbae* to host DNA to establish infection level (Appendix B). However, it is more accurate and often more cost effective to analyze blood parasite infection intensity by examination of the thin blood smears and counting the number of parasites per 100 microscopy fields at 1000x, which is an accepted method (Sol et al. 2003, Waite et al. In Press).

Blood was also taken from the brachial vein for Enzyme-linked Immunosorbent Assays (ELISAs), but it was collected directly from the vein into 1.5 mL Eppendorf

tubes, rather than into capillary tubes. Tubes were then closed, held in one hand, and flicked using the other hand to agitate the blood and prevent large clots from forming. All blood collected for ELISAs was immediately put onto ice following collection, then spun for 10 minutes at 10,000 rpm to separate blood cells from serum. The top serum layer was collected from the spun-down blood, put into an o-ring microcentrifuge tube for storage, and both serum and whole blood cell layers were put into freezer storage at -20 °C for later use. Details of the ELISA protocol are provided in Chapter 3.

1.4 Specific Aims and Chapter Summaries

I begin this dissertation with the interaction between host and vector, which is the overriding topic of Chapter 2 and Chapter 3. This first initial interaction is critical to the host becoming infected with parasites in a vector-borne parasite system, yet much research has focused on preventing the vector from reaching the host (insecticides, bednets, repellents, etc) rather than trying to understand what happens at this encounter. Chapter 2 describes the behavioral defenses of pigeons against the hippoboscid fly, and quantifies their effectiveness against flies (Waite et al. 2012). Chapter 3 looks at two defenses against the fly: behavioral defense and immunological defense and whether the defenses are more effective in combination. Multiple host defenses can be important in shaping the ecology and evolution of both pathogens and their hosts (Parker et al. 2011).

In Chapter 3 I show that behavioral and immunological pigeon defenses are each effective against flies, and that the two defenses work additively. This result was somewhat unexpected as it has been shown that using one defense allows an organism to downregulate another, presumably to conserve energy (Castella et al. 2008), and that defenses can work synergistically, where the combination of two defenses is actually

greater than the additive effect of each defense by itself. Such synergistic effects are often seen in application of pesticides in agricultural settings (Miller et al. 1983). Immune responses in pigeons could conceivably direct preening to particular parts of the body, making this behavior more effective, yet I found no such effect. There was also no impact of these anti-vector defenses on reducing malaria prevalence or infection intensities in the birds. This is possibly because the defenses did not act quickly enough or severely enough against the flies. It remains to be tested whether the results of this experiment would be the same if flies were allowed to choose between hosts with variable defenses.

In Chapter 4 I describe the results of a field experiment where we examined the reciprocal effect of parasite on host (Knutie et al. 2012). We tested the effect of malaria parasites on very young nestling pigeons. Nestlings were inoculated with malaria parasites in an effort to isolate effects of the malaria by using an experimental approach, rather than through correlations of infection and fitness where results can be harder to interpret. Chronic *H. columbae* infection is correlated with decreases in pigeon survival, notably more so for pigeons under six months of age (juvenile birds) (Sol et al. 2003). However, selection on differences in parasite intensity between adult and juvenile birds could not fully explain the difference in survival between these two age classes (Sol et al. 2003). If the nestling (or younger than "juvenile") pigeons experienced even greater impacts of the malaria, this would perhaps fully explain the differences between older and younger birds in parasite load by selection alone. Surprisingly, we found no effect of the malaria parasites on nestling pigeons. Birds injected with malaria parasites, with a control, or birds not injected did not differ significantly in growth, survival, fledging success, or postfledging survival. We suggest nestlings may have greater tolerance to

malaria than adult pigeons. Nestling pigeons are cared for by both parents, and are fed a rich diet of crop milk full of fat and protein. Parents continue to supplement the diet of offspring after they fledge for several weeks (Johnston and Janiga 1995).

While the effects of the malaria parasite on the pigeon are an interesting story, it is a story about only half of the parasite's life cycle. Equally, if not more important, is the sexually reproductive stage of the parasite, which takes place in the fly. Chapter 5 describes the interaction between the pigeon malaria parasite and its hippbosocid fly vector (Waite et al. In Press). The effect of malaria parasites on their vectors is a topic that has been somewhat controversial and muddied in the past, with the chief problems stemming from conflicting work using unnatural vector-parasite combinations and from differences in how long experiments were run (Ferguson and Read 2002). We used the natural pigeon-fly-malaria parasite system to test whether the flies were affected by feeding on malaria-infected pigeons over the life span of the adult flies.

This is a unique system where the relative impacts of malaria parasites on male and female vectors can be compared since both fly sexes act as vectors. We found that female flies, but not males, had lower survival when they fed on malaria-infected birds. Malaria also reduced female reproduction. We suggest that the greater investment in reproduction by females explains these results. Females also take larger blood meals than males (related to their larger reproductive costs) and may therefore be exposed to a larger number of parasites. The results of feeding trials suggest that even though *H. columbae* may use resources from the blood (Manwell and Loeffler 1961), and thus might be in competition with the fly for resources, that the nutritional quality of the blood meal between infected and uninfected pigeons does not affect short term survival for either

male or female flies. Flies that fed on either infected or uninfected blood survived equally long away from the host.

1.5 Conclusions

In conclusion, this work experimentally tests the fitness consequences of the interactions among organisms in a host-vector-parasite system using Rock Pigeons, hippoboscid flies, and a pigeon malaria parasite. I find that pigeons have multiple defenses that reduce fly survival and reproduction, and that these defenses work additively against flies. By lowering vector populations, pigeons would also lower the likelihood that the malaria parasite is transmitted at a population level. I also show that nestling pigeons are not harmed by malaria infection at this young age, possibly because they are more tolerant and are in better body condition than older fledged birds. I show that flies are harmed by the malaria parasite, but that only female survival and reproduction is affected with no significant impact on male flies. This might suggest that male flies are better vectors than females since the immense cost of reproduction in conjunction with malaria infection decreases female fly survival significantly. However, females are only affected fairly late in their life, and typically take larger blood meals than males, which might suggest the opposite. More studies are needed, such as on the relative frequency of movement between hosts, and the number of parasites that male and female flies each transmit, to determine their relative value as vectors of malaria parasites. Collectively this work can be used to understand the relative importance of different interactions between organisms in vector transmitted parasite systems to better understand the evolution of all of the organisms involved.

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CHAPTER 2

HOW EFFECTIVE IS PREENING AGAINST MOBILE

ECTOPARASITES? AN EXPERIMENTAL TEST

WITH PIGEONS AND HIPPOBOSCID FLIES

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How effective is preening against mobile ectoparasites? An experimental test with pigeons and hippoboscid flies

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ABSTRACT

Birds combat ectoparasites with many defences but the first line of defence is grooming behaviour, which includes preening with the bill and scratching with the feet. Preening has been shown to be very effective against ectoparasites. However, most tests have been with feather lice, which are relatively slow moving. Less is known about the effectiveness of preening as a defence against more mobile and evasive ectoparasites such as hippoboscid flies. Hippoboscids, which feed on blood, have direct effects on the host such as anaemia, as well as indirect effects as vectors of pathogens. Hence, effective defence against hippoboscid flies is important. We used captive Rock Pigeons (*Columba livia*) to test whether preening behaviour helps to control pigeon flies (*Pseudolynchia canariensis*). We found that pigeons responded to fly infestation by preening twice as much as pigeons without flies. Preening birds killed twice as many flies over the course of our week-long experiment as birds with impaired preening; however, preening did not kill all of the flies. We also tested the role of the bill overhang, which is critical for effective preening against feather lice, by experimentally removing the overhang and re-measuring the effectiveness of preening against flies. Birds without overhangs were as effective at controlling flies as were birds with overhangs. Overall, we found that preening is effective against mobile hippoboscid flies, yet it does not eliminate them. We discuss the potential impact of preening on the transmission dynamics of blood parasites vectored by hippoboscid flies.

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1. Introduction

Birds are infested with a variety of ectoparasites including lice, mites, ticks, fleas and flies, all of which have the capacity to decrease host fitness (Atkinson et al., 2008; Møller et al., 2009). Birds combat ectoparasites with defences ranging from anti-parasite behaviour (Hart, 1992, 1997) to immune defences (Wikel, 1996; Owen et al., 2010). Grooming behaviour, which includes preening with the bill and scratching with the feet, is the first line of defence against ectoparasites (Clayton et al., 2010). Preening is an energetically expensive activity (Goldstein, 1988; Croll and McLaren, 1993); furthermore, the time and energy devoted to preening detracts from other behaviours such as feeding and vigilance (Redpath, 1988). Therefore, in order to be effective against ectoparasites while limiting its energetic cost, preening should be an inducible defence (Tollrian and Harvell, 1999). The importance of preening is illustrated by recent work demonstrating that features of bill morphology, such as the upper mandibular overhang, appear to have evolved specifically to enhance the effectiveness of preening for parasite control (Clayton and Walther, 2001; Clayton et al., 2005).

Nearly all of the work on the effectiveness of preening has been done with feather lice (Phthiraptera: Ischnocera), which are slow moving and therefore relatively easy targets for preening birds (Marshall, 1981; Atkinson et al., 2008). The effectiveness of preening for controlling more mobile ectoparasites such as fleas and hippoboscid flies has not, to our knowledge, been tested. Preening may also play a role in shaping vector ecology and the evolution of pathogens transmitted by ectoparasites.

The goal of our study was to test the effectiveness of preening against hippoboscid flies, which are mobile parasites of birds and mammals. Avian hippoboscid flies are dorso-ventrally flattened and very agile at slipping between the feathers. As described by Rothschild and Clay (1952): "They have... an extremely efficient method of moving among feathers – darting and scuttling about at a remarkable speed – and are extremely difficult to catch on a living bird." Hippoboscids may also be capable of avoiding preening by using "refugia" such as the vent region of the bird or behind the bases of the legs (Waite, personal observation).

Hippoboscid flies are a diverse group of parasites. More than 200 species are recognised, 75% of which parasitise birds belonging to 18 orders; the rest parasitise mammals (Lloyd, 2002; Lehane, 2005). Most species of bird flies are winged and capable of flight between individual hosts (Harbison et al., 2009; Harbison and Clayton, 2011). They spend most of their time on the body of the

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bird, where they feed on blood several times a day (Coatney, 1931). Hippoboscid feeding can cause anaemia (Jones, 1985), emaciation (Lloyd, 2002) and slow nestling development (Bishopp, 1929). Parents of hippoboscid-infested nestlings have lower reproductive success (Bize et al., 2004). Hippoboscid flies also transmit blood parasites that can have negative effects on birds, including malaria (Sol et al., 2003), trypanosomes (Baker, 1967) and possibly viruses such as West Nile (Farajollahi et al., 2005). In short, hippoboscids pose both direct and indirect threats to the health and fitness of their hosts.

To test the effectiveness of preening against hippoboscid flies, we used wild caught Rock Pigeons (*Columba livia*) that we experimentally infested with the pigeon fly *Pseudolynchia canariensis* (Diptera: Hippoboscidae). We conducted two separate experiments. The first experiment addressed two questions: (i) do Rock Pigeons infested with flies increase the amount of time they spend preening and (ii) is preening effective in killing flies? The second experiment addressed a third question: is the bill overhang important in the effectiveness of preening for fly control?

2. Materials and methods

2.1. Experiment 1: preening and flies

Twenty-four Rock Pigeons were caught using walk-in traps in Salt Lake City, Utah, USA. The birds were transported to the University of Utah animal facility, where they were individually housed in wire mesh cages (30 × 30 × 56 cm) suspended over newspaper-lined trays. Each cage/tray was completely enclosed within a fly-proof net, which prevented flies from moving between birds in different cages. Birds were given ad libitum food, water and grit and kept in a 12-h light/dark cycle. They were maintained in captivity for at least 6 months at low humidity prior to the experiment, which killed feather lice and their eggs that were present on the birds when they were captured (Harbison et al., 2008). Any flies present on pigeons when they were captured would have died during the 6 month period because the life span of pigeon flies is only 2–3 months (Fahmy et al., 1977). Since pigeons trapped in Salt Lake City do not usually have other ectoparasites, the birds were ectoparasite-free at the start of our experiment. Prior to the start of the experiment, birds were carefully examined to confirm that they did not, in fact, have any ectoparasites.

We blocked the 24 birds using two factors: (i) location trapped and (ii) time in captivity; we then randomly assigned birds to one of three treatments, with eight birds per treatment. All birds were sexed and weighed. Birds in the first two treatments were then infested with 20 flies each (10 male flies, 10 female flies), which is the maximum number recorded from wild pigeons (mean = 5.07 flies; Stekhoven et al., 1954). Flies used to infest birds were cultured from wild caught stock on pigeons kept for this purpose in another room. The third group of eight birds was not infested with flies.

Flies were removed from culture birds using CO_2 (Moyer et al., 2002). They were sexed under a microscope at 25× before putting them on experimental birds. Half of the birds (chosen at random) in each of the two fly-infested treatments had plastic attachments fitted to their bill to impair their ability to preen. The attachments are small C-shaped pieces of plastic that, when fitted in the nares of a pigeon, create a 1.0–3.0 mm gap between the mandibles. This gap prevents the full occlusion of the bill needed for effective preening (Clayton et al., 2005). The attachments are harmless; they do not impair feeding or alter the amount of time that birds attempt to preen (Clayton and Tompkins, 1995; Koop et al., 2011).

To address our first question whether pigeons preen more when they are infested with flies, we compared the behaviour of birds with normal (unimpaired) preening with and without flies. Preening

behaviour was quantified using instantaneous scan sampling between 13:00 and 16:00 h (Altmann, 1974). Preening was defined as touching the plumage with the bill (Clayton and Cotgreave, 1994). Birds were observed at 6 s intervals (Clayton, 1990) for 30 observations per bird per day, for 5 days following infestation. We calculated the proportion of time that birds spent preening.

To address our second question whether preening is effective in killing flies, we compared the number of flies killed by birds with impaired preening with flies killed by birds with normal preening. The experiment lasted 1 week, after which one of the authors (Waite) removed dead flies from the bottom of each cage; food and water dishes were also checked for dead flies. Another author (Henry) re-examined all cages to ensure that nothing was overlooked. Damage to flies was observed and recorded under a microscope at 25×. Flies were scored as preening-damaged if the head, thorax, abdomen or at least one wing was crushed or missing, or if at least three legs were missing. We calculated the proportion of flies with preening-damage out of the total number of dead flies recovered for each host after 1 week.

2.2. Experiment 2: bill overhang

Another 12 wild-caught (individually caged) pigeons were used for this experiment. Birds were again blocked by location trapped and time in captivity. Half of the birds, chosen at random, had their bill overhang trimmed away with a dremel tool. The other half was sham trimmed, i.e. they were handled but no part of the bill was removed (Fig. 1). The trimming method, which is fully described in Clayton et al. (2005), does not harm the birds in any way. One week after trimming (or sham trimming) all birds were sexed and weighed, and then each bird was infested with 20 flies (10 males, 10 females). Preening behaviour and fly mortality were quantified as in Experiment 1.

2.3. Statistical analysis

Statistical analyses were performed in Prism® v. 5.0b (GraphPad Software, Inc.). Data were analysed using Mann–Whitney U Tests
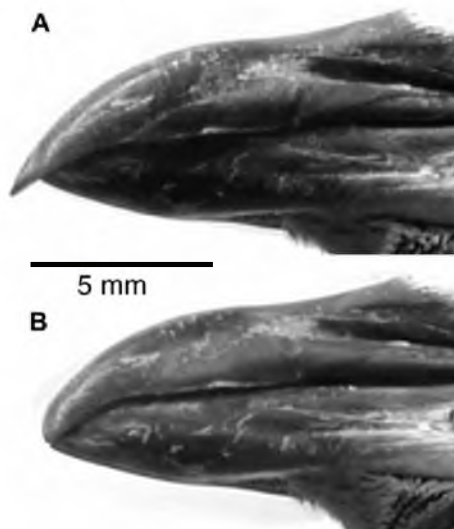
Fig. 1. Rock Pigeon bill showing upper mandibular overhang before (A) and after (B) removal of the overhang. The overhang grows back after several weeks. Figure reproduced from Clayton et al. (2005).

for comparisons between two groups. ANOVAs were used for comparisons among three groups. The sex ratio of pigeon hosts in each experiment was compared using a Chi-square or Fisher's Exact test, as appropriate. Values are presented as mean ± S.E. Results were considered significant at $P \leqslant 0.05$.

3. Results

Sex and body mass of hosts did not differ significantly by treatment in either experiment (Experiment 1: sex, Chi-square test, $P = 0.77$; mass, ANOVA, $F_{2,21} = 1.47$, $P = 0.25$; Experiment 2: sex, Fisher's Exact test, $P = 1.00$; mass, Mann–Whitney $U = 12.5$, $P = 0.42$).

3.1. Experiment 1: preening and flies

Birds infested with flies preened more than twice as much as birds without flies; birds with flies preened 23.49 ± 3.96% of the time observed, whereas birds without flies preened 11.21 ± 2.11% of the time observed; (Fig. 2). The difference in preening rates between the two groups was statistically significant (Mann–Whitney $U = 10.5$, $P = 0.03$).

Birds with normal preening killed twice as many flies as birds with impaired preening; birds with normal preening killed 43.75 ± 5.41% of flies, compared with 21.88 ± 5.74% of flies killed by birds with impaired preening (Fig. 3A). The difference in the number of flies killed was statistically significant ($U = 11.0$, $P = 0.03$).

Birds with normal preening also damaged a significantly greater proportion of dead flies than did birds with impaired preening (Fig. 3B; Mann–Whitney $U = 7.0$, $P = 0.01$). Of the dead flies recovered from normally preening birds, 44.6 ± 0.06% were damaged, while only 16.6 ± 0.13% of flies recovered from birds with impaired preening were damaged.

3.2. Experiment 2: bill overhang

Removal of the bill overhang had no significant effect on preening time; birds without overhangs preened 12.96 ± 1.08% of the time observed, while birds with overhangs preened 16.81 ± 3.90% of the time observed (Mann–Whitney $U = 13.0$, $P = 0.47$). Birds with overhangs did not kill significantly more flies than birds with no overhang; birds with overhangs killed 50.83 ± 11.93% of flies, compared with 45.00 ± 11.76% of flies killed by birds with no overhang (Fig. 4; Mann–Whitney $U = 15.0$, $P = 0.69$). Thus, the bill overhang was not a factor in the efficiency with which preening killed flies.

Fig. 3. Effect of preening and an example of preening damage. (A) Proportion of flies killed by birds with normal versus impaired preening. (B) Example of intact versus preening-damaged flies.

Fig. 2. Proportion of time that birds with and without flies spent preening.
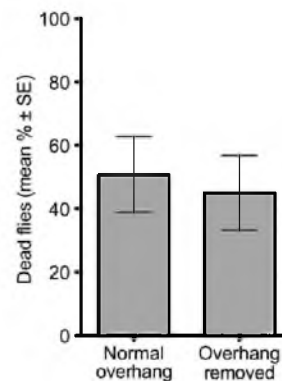
Fig. 4. Proportion of flies that were dead in cages of birds with and without bill overhangs.

4. Discussion

We examined the effectiveness of preening against mobile ectoparasitic flies. Pigeons experimentally infested with flies preened twice as much as pigeons without flies (Fig. 2). Preening also

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proved to be effective against flies (Fig. 3A); we recovered twice as many dead flies from the cages of birds that could preen, compared with those that could not preen. Pigeons were able to catch and crush flies (Fig. 3B), even though the flies are extremely adept at moving quickly and evasively through the feathers (Rothschild and Clay, 1952).

Removal of the bill overhang did not decrease the efficiency of preening significantly (Fig. 4). Clayton et al. (2005) showed that lice are crushed when birds preen by the mortar-and-pestle action of the tip of the lower mandible moving against the upper mandibular overhang. Although the overhang is essential for controlling feather lice, our results show that it is not needed when preening flies, presumably because the flies are much larger and softer-bodied than lice. Although preening proved to be an effective defence against flies, it did not eliminate all of them over the course of our week-long experiment. Only one of 40 birds in the two experiments cleared itself completely of flies.

Preening may have the added benefit of helping to protect birds from pathogens for which the flies are vectors. In principle, preening can prevent transmission of pathogens if it kills infected vectors before they have an opportunity to bite the host. The fly *P. canariensis* is a known vector of the blood parasites *Haemoproteus columbae* and *Trypanosoma hannae* (Fahmy et al., 1977; Mandal, 1991). Waite (unpublished data) recently showed that pigeons exposed to just five flies for 3 days can become infected with *H. columbae*. In our study, only an average of 50% of flies placed on pigeons were killed during the week-long experiment (Fig. 3A). Thus, even birds with relatively efficient preening may remain at risk of acquiring blood parasites. If preening irritates flies, encouraging them to move between hosts, then preening might even have the effect of increasing pathogen transmission (Hodgson et al., 2001). It would be very interesting to measure the impact of preening on pathogen transmission by hippoboscid flies among birds in a population.

We found that pigeons infested with flies doubled the amount of time that they spent preening compared with controls (without flies) and compared with the typical rates of preening for other pigeons and doves (Clayton, 1990; Koop et al., 2011). One might predict that experimental birds would spend even more time preening, given that they did not completely remove their infestations in most cases. However, research on the cost of preening shows that it is energetically expensive. When birds preen, their metabolic rates increase by as much as 200% (Wooley, 1978; Croll and McLaren, 1993). The energetic cost of preening might explain why preening is an inducible defence against hippoboscid flies. Additional indirect costs of preening include the time taken away from courtship behaviour, foraging and predator surveillance (Redpath, 1988). Thus, in addition to the direct impact of hippoboscid flies on host fitness, flies may have indirect effects mediated by the energetic and time related costs of preening. Indeed, there may well be a trade-off between the indirect cost of preening and the more direct costs of fly infestation.

Acknowledgements

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CHAPTER 3

EFFECTS OF BEHAVIORAL AND IMMUNOLOGICAL

DEFENSES AGAINST VECTORS OF

AVIAN BLOOD PARASITES

3.1 Abstract

Blood-feeding arthropods can harm their hosts through many mechanisms, such as by causing direct tissue damage/anemia, distracting hosts from foraging or surveillance activities, and by transmitting pathogens. Thus, effective behavioral and immunological defenses against blood-feeding arthropods may provide important fitness advantages to hosts if they reduce bites, or in systems involving pathogen transmission, if they lower pathogen transmission rate. We quantified the effectiveness of behavioral and immunological defenses of Rock Pigeons *(Columba livia)* exposed to blood-feeding hippoboscid flies *(Pseudolynchia canariensis)* infected with the pigeon malaria parasite *(Haemoproteus columbae)*. Birds were subjected to four treatments in which they: 1) could preen and were immunologically "primed" against flies, 2) could not preen but were primed against flies, 3) could preen but were not primed, or 4) could not preen, nor were they immunologically primed. We found that both defenses were effective in decreasing the survival and reproductive success of flies. However, these defenses did not reduce malaria parasite transmission. The intensity of donor infections was positively correlated with the malaria parasite intensity in experimental hosts, although this only

explained a portion of the variation. Malaria parasite genetics may also play a role in transmission dynamics, though the role of genetic diversity remains to be tested in this system.

Keywords: malaria, arthropod saliva, *Haemoproteus*, virulence, transmission

3.2 Introduction

Blood-feeding arthropods and the parasites they transmit are important components of the diversity of infectious diseases that affect human health, animal agriculture and wild species (Sachs and Malaney 2002, Arcoverde et al. 2009). When arthropods bite, at a minimum they may cause irritation or distract their host from feeding or surveillance activities; they can also cause anemia, inflammation, and scabbing (Wall 2007). This interaction between host and arthropod represents a key bottleneck in the transmission of blood-borne pathogens and, as such, it is potentially a target for disease control (Sinden 2010).

Along with transmitting parasites, arthropods (vectors) transmit many salivary compounds when they feed that enhance acquisition of host blood. These compounds can affect the physiological conditions at the bite site, blocking hemostasis, causing vasodilation, and reducing inflammation (Adie 1915, Champagne 2004). Some parasites have co-evolved with their vectors to take advantage of these vector effects for their own successful establishment. For example *Leishmania* species are more virulent when vectored by sandflies than when injected without sandfly salivary compounds, in particular the salivary compound maxadilan (Bishopp 1929, Titus et al. 2006). Similarly, the virulence of malaria parasites is enhanced by the presence of mosquito saliva (Herath 1966, Klei 1971, Alger et al. 1972). However, these same salivary compounds can also

be potent antigens that stimulate the host immune defense to act against the vector (Barral et al. 2000, Andrade et al. 2005). In some cases pre-exposure to uninfected saliva protects the host from vector-transmitted pathogens in these same disease systems (Alger et al. 1972, Gomes et al. 2008).

In nature, vertebrate hosts can and do defend themselves against vectors, using behavioral defenses (e.g. grooming) (Clayton and Tompkins 1995, Koop et al. 2012) and immunological defenses (Owen et al. 2010). Anti-vector defenses may affect pathogen transmission either directly or indirectly. Behavioral defenses may directly stop pathogen transmission by preventing bites, or decreasing feeding duration or meal size. For example, ciconiiform birds with more defensive behavior are more effective in preventing mosquitoes from feeding on them (Adie 1924, Edman and Kale 1970), and the same is true for passerine and galliform species (Darbro and Harrington 2007). Behavioral defenses may have indirect benefits if the energetic costs to the host associated with reducing vector fitness is offset by the benefits of smaller vector populations and potentially a reduced rate of pathogen transmission. The same potential trade-offs may also exist for immune defenses against vectors.

Immune defenses against vectors may stop pathogen transmission by preventing the necessary vector-induced physiological changes at the bite site that promote blood-feeding, or indirectly stop transmission by reducing the overall number of vectors (higher mortality and/or lower fecundity). Immune defenses against longer-term feeders, such as ticks, were discovered decades ago (Trager 1939, Moyer et al. 2002). The idea that the immune system can also protect against shorter-term feeders (e.g. sandflies and mosquitoes) is less intuitive, but in some cases immune defenses against short-term

feeders can also be effective. Immunoglobulins to salivary compounds can decrease feeding, thus acting within minutes (Ahmed and Mohammed 1978b, Milleron et al. 2004), and proteolytic compounds released by basophils and eosinophils ingested with the blood meal can tear apart the gut of feeding vectors, acting later as the meal is digested (Ahmed and Mohammed 1978a, Wikel 1996).

It is conceivable that different types of anti-vector defenses interact, enhancing the effectiveness of each (synergistic interaction), or possibly reducing the effectiveness of each (antagonistic). Alternatively, they may work additively, i.e. the combination of defenses has no impact on their individual effectiveness. The effect of anti-vector defenses in natural host-vector-pathogen systems is poorly understood, but exploring these effects will enhance our understanding of the ecology and evolution of infectious disease and may suggest new avenues for disease control.

The goals of this study were to (1) test the effectiveness of both host behavioral and immunological defenses against vectors, (2) determine the nature of any interaction between them, and (3) test whether anti-vector defenses decrease the transmission and virulence of the parasites being vectored in a natural disease system. To do this we chose a disease system consisting of wild caught pigeons (Rock Pigeon, *Columba livia*) the pigeon malaria parasite *Haemoproteus columbae*, and a hippoboscid fly vector (*Pseudolynchia canariensis*) that feeds on pigeon blood.

The effect of *H. columbae* on wild pigeons is chronic, leading to a gradual reduction in survival (Adie 1924, Sol et al. 2003), with generally mild effects in captivity (Acton and Knowles 1914, Coatney 1933). *H. columbae* reproduces asexually in the avian host, and sexually in the vector. Both male and female flies take frequent blood meals, feeding

twice daily for 20-80 minute bouts (Arcoverde et al. 2009). *H. columbae* is transmitted by both fly sexes, which is unusual for a malaria parasite. *H. columbae* reaches maturity after 10 days, then migrates to the salivary glands of the fly to be transmitted to another pigeon (Adie 1915). The fly life cycle is also unusual in that one egg hatches *in utero* and the subsequent three larval stages take place inside the female before she deposits the pupated offspring. *P. canariensis* females produce one puparium at a time every 2-3 days after reaching sexual maturity (6 days of age) (Herath 1966, Klei 1971). They usually place puparia in or around pigeon nests (Bishopp 1929), but will deposit them under layers of newspaper lining pigeon cages in captivity. The flies appear to be irritating to the pigeons because infested birds double preening activity (Waite et al. 2012a).

We tested three specific hypotheses using this system: (1) Host behavioral and immunological defenses decrease fly fitness, specifically reducing survival and/or fecundity (2) Host behavioral and immunological defenses interact synergistically against flies, and (3) Host defenses against the vector reduce *H. columbae* transmission.

3.3 Methods

3.3.1 Pigeons and Treatment Groups

Pigeons were bred in captivity to generate birds with no previous exposure to flies or malaria parasites. All pigeons were bred from feral adults caught with walk-in traps at sites in Salt Lake City, UT. Young pigeons were hatched between July 2008 and February 2009, and were mature (≥6 months old) at the start of the experiment in December 2010. The experiment was run in a two by two factorial design (Figure 3.1) with four treatments. The four treatments were as follows: A) birds could preen and were immunologically "primed" against flies, B) birds had their preening impaired, but were

immunologically primed against flies, C) birds could preen but were not immunologically primed, and D) birds were preening impaired and not primed. The experiment was replicated 12 times (N = 48 pigeons total) with treatment group randomly assigned after first assigning siblings to the same replicate when possible with one sibling in each treatment. This was done to help control for any parental effects on defense.

Pigeon immune system priming against flies for half of the pigeons was conducted during the two weeks prior to the experiment to allow enough time for a specific IgY antibody response (Davidson et al. 2008). All pigeons were fitted with fly backpacks (Figure 3.2) for the two weeks before the experiment. Pigeons had their immune systems "primed" (Figure 3.1, treatments A,B) against flies by exposure to 10 recently eclosed flies (≤ 2 days old) in their backpack; those that remained naïve to flies (C,D) had empty backpacks for this 2-week period. Feathers in the 3cm x 3cm region of the backpack were carefully removed to allow flies to feed; feathers were also removed from control birds that wore backpacks without flies. Backpacks were removed after 2 weeks. Half of the birds (chosen at random) in each of these backpack treatments were fitted with harmless "bits" (B,D) to impair their ability to preen flies (Waite et al. 2012a). In the preening impaired birds bill mandibles were trimmed weekly to prevent the mandibles from growing back to fully occlude around the bits over the 5 weeks of the experiment.
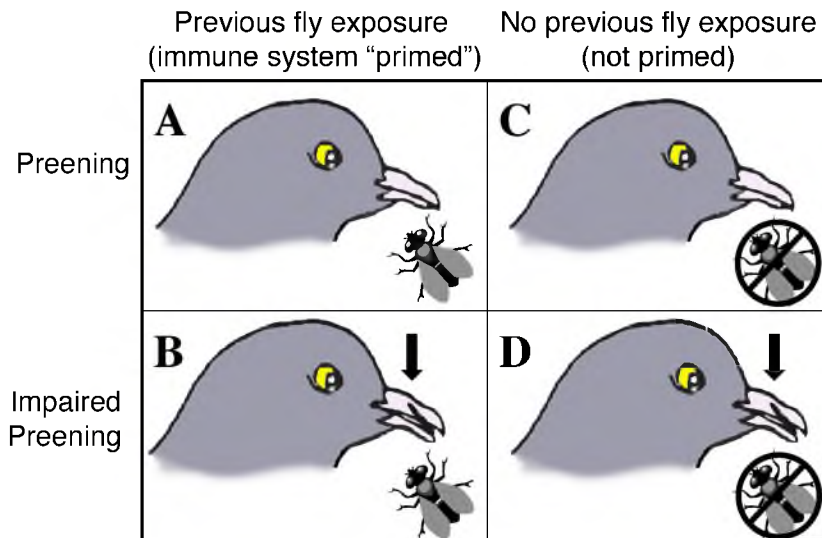
Figure 3.1. A 2x2 factorial design to test the effectiveness of behavioral and immunological defenses - and any interaction between them - against flies. Half of the birds (A,B) had their immune systems primed against flies by exposure to flies in a backpack for three weeks prior to the start of the experiment (Figure 2). The other half (C,D) wore backpacks with no flies. Preening was normal (A,C) or impaired (B,D).

Figure 3.2. Pigeon wearing a backpack with elastic straps around the wings. Mesh netting on the bottom allowed flies to feed on the pigeon's back where feathers have been removed (Mohammed 1958). Pigeons could not remove flies from the backpack by preening.

3.3.2 Flies

Flies used in the backpacks had not previously been fed. The malaria parasite *Haemoproteus columbae* is not transmitted from flies to their offspring, and so it was certain the flies used in backpacks were uninfected. Flies in the backpacks were used only for immunological priming and not later in the experiment.

New groups of 10 freely moving flies were added to each bird in all treatments following the immunological priming period, just after backpacks were removed and birds in the preening impaired treatments had been bitted. Each bird received five male and five female flies (sexed under a microscope at 25x magnification). Each replicate of four birds received flies from the same cohort. Each cohort of 40 flies was exposed to malaria parasites before flies were placed on experimental birds. For each replicate (N = 12 replicates) 40 unfed flies ($\leq$ 2 days old) were placed on a single naturally infected wild-caught pigeon infected with malaria parasites (intensity range 52 – 612, mean 273 parasites in 100 microscope fields examined at 1000x, N = 12 "donor" birds, one for each replicate). All cohorts of flies were left on the donor pigeon for 12 days, allowing *H. columbae* to reach the infective sporozoite stage in the fly salivary glands (Adie 1924), then flies were collected from the donor birds and ten flies were transferred to each of the experimental (captive bred) pigeons. Experimental pigeons were kept in cages enclosed in fly-proof netting. Thus, each bird received five male and five female flies of the same age with the same exposure to malaria parasites for the birds in each treatment.

Dead flies and puparia were removed weekly from cages to track fly survival and reproduction; for further methods see Waite et al. (2012a). After five weeks of the experiment all flies were removed from the cages and from the birds using a combination

of CO_2 exposure for 12 minutes (Moyer et al. 2002), followed by three minutes of ruffling feathers of the birds over a white table to collect flies. Five weeks time was chosen because the primary goal of this experiment was to observe the effect of host defenses on fly fitness, and this time encompasses the average lifespan of an adult fly in captivity (Waite et al. 2012b). Birds were also prevented from being reinfected with *H. columbae* by removing flies at this point in the experiment. This is because the life cycle of *H. columbae* takes longer than 35 days to complete transmission. First parasites must develop in the bird's peripheral blood to mature transmissible stages (typically 35 days) and then develop in the fly to the sporozoite stage (10-12 days) for it to be possible for a bird to be reinfected (Adie 1924, Ahmed and Mohammed 1978a, 1978b).

3.3.3 Blood Samples

Blood was sampled every three days from day 21 to 70 of the experiment, with blood smears made each time. Smears were stained with Giemsa (diluted with buffer 1:10, pH 7.0, 50 minutes) and examined under oil immersion at 1000x for 10 minutes; if parasites were detected in a sample, then the number of parasites was quantified in 100 unique microscope fields filled with nonoverlapping blood cells.

3.3.4 Immunology

Additional blood samples were taken to measure IgY antibody levels in pigeon blood serum. The first blood sample was taken just prior to fitting pigeons with backpacks to measure baseline *P. canariensis*-specific antibody levels. Subsequent samples were taken weekly up to 5 weeks (day 35 of the experiment). Samples were collected directly from the brachial vein into a 1.5ml eppendorf tube, flicked 3 to 5 times to prevent large clots from forming, and immediately put on ice. Samples were spun at 10,000 rpm for 10

minutes to separate blood cells from blood serum, and the serum layer pipetted into a 1 ml O-ring sealed microcentrifuge tube, then stored at -20°C. Blood serum was analyzed using an indirect ELISA following the methods of Huber et al. (2010).

Briefly, 96-well Nunc-MaxiSorp flat bottom ELISA plates were coated in triplicate with 100 μl of *P. canariensis* extract diluted at 1:100 in carbonate coating buffer (0.05M, pH 9.6, Sigma). Plates were incubated for 1 hour at room temperature on an orbital table, or overnight at 4°C, and then washed five times with wash buffer (tris-buffered saline with Tween 20, Sigma). Wells were then coated with 200 μl bovine serum albumin (BSA) blocking buffer, incubated for 30 minutes at room temperature on an orbital table, and then washed five times with wash buffer. Each well was then loaded with 100 μl of pigeon serum diluted 1:100. Plates were incubated for one hour at room temperature on an orbital table and then washed five times with wash buffer. Next 100 μl of Goat-anti-Bird-IgY (1:5000) were added to each well, incubated at room temperature on an orbital table for one hour, and then washed five times. Finally, 100 μl of peroxidase substrate (tetramethylbenzidine, TMB: KPL Laboratories cat. 50-76-00) were added to each well. The plates were incubated for exactly 10 minutes at room temperature and the reaction was stopped using 100 μl of 2 M H_2SO_4 in each well, before reading optical density on a spectrophotometer using a 450-nanometer filter. On each plate we included three wells for non-specific binding, which quantified binding of Goat-anti-Bird-IgY to the antigen. These wells received all of the reagents described above with the exception of pigeon serum. In this step, blocking buffer was used in place of serum. We also included three wells that were positive controls where a pooled sample of pigeon serum was used on all of the plates that were run so that samples could be compared across plates. We

additionally included three blank wells, which received the reagents except for the antigen and serum steps where either plain coating buffer or blocking buffer was used respectively. The mean absorbance of the NSB wells on each plate was subtracted from the absorbance measures determined above for each of the samples. Finally, we calibrated absorbance values between plates using a positive control. The reference sample absorbance was compared across all plates, and we calculated a correction factor for each plate to standardize absorbance.

3.3.5 Statistical analysis

Statistical analyses were carried out using Prism v. 5.0d (GraphPad Software, Inc.). Survival analysis using a Cox proportional hazard model was run in R version 2.13.0 (R Development Core Team 2011) with the survival package (Therneau and Lumley 2011). Because malaria is known to have different effects on males and females (Waite et al. 2012b), most fly data were analyzed by sex.

3.4 Results

Immunologically "priming" pigeons against flies by exposure to uninfected flies significantly increased their anti-fly antibody levels compared to naïve controls (Figure 3.3; t-test, t = 3.653, df = 45, P = 0.0007). However, birds that began the experiment naïve to flies "caught up" in their anti-fly IgY antibody levels after 2 weeks of exposure to infected flies in the experiment, and were indistinguishable from "primed" birds at this point (ANOVA $F_{3,47}$ = 0.842, P = 0.478).
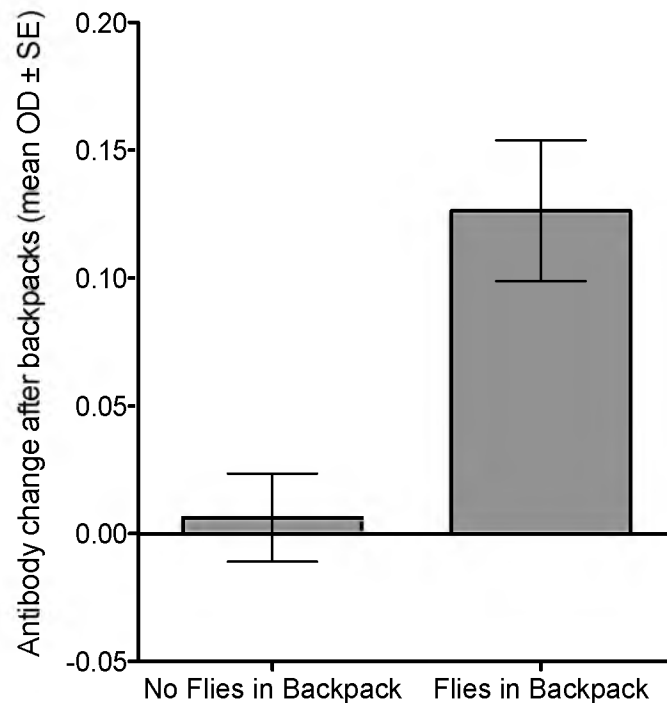
Figure 3.3. – Fly-specific IgY antibodies increased significantly in birds preexposed to flies for 2 weeks compared to birds that were previously unexposed to flies (see text for statistics). All birds were fitted with backpacks (see Figure 3.2); half of the backpacks had ten uninfected flies to "prime" the anti-fly immune response, the other half had no flies.

There was no significant interaction between preening and immune defenses on fly survival (Cox proportional hazards (PH) test for interaction of preening*immune exp(coef) = 0.763, P = 0.18). Therefore, fly survival data were analyzed with both preening and immune defenses as factors without the interaction term. In this preening+immune model, preening and immune defenses were each effective in decreasing fly populations over the course of 5 weeks; preening increased fly mortality rate by 33.4%, and immunological priming increased fly mortality rate by 31.7% (Cox PH preening+immune model, preening P < 0.0001, exp(coef) = 0.334, immune P = 0.007, exp(coef) = 1.317). When fly sexes were analyzed separately, again there was no

significant interaction between the defenses for either female or male fly survival (females; preening*immune P = 0.22, exp(coef) = 0.709; males; preening*immune P = 0.18, exp(coef) = 0.656). Analyzing the effect of defenses as factors for each fly sex revealed that female survival was significantly decreased by both defenses (Figure 3.4; Cox PH preening P < 0.0001, exp(coef) = 0.338, immune P = 0.0016, exp(coef) = 1.845), but male survival was only decreased by preening, without a significant impact of host immune response (Cox PH preening P = 0.0007, exp(coef) = 0.486, immune P = 0.16, exp(coef) = 1.359). The increase in mortality rate due to preening defense was similar between the fly sexes (females experienced a 33.8% increase in mortality due to preening, males 48.6% increase compared to flies on preening impaired birds). The immune defense increased female fly mortality by 15.5% compared to that of females on birds that had not had prior immune priming with flies.

Female flies on preening birds produced fewer puparia per capita on average than those on bitted birds over the first 2 weeks of the experiment; however, there was no significant effect of immune defense on the number of puparia, nor was there a significant interaction (Figure 3.5; two-way ANOVA, preening $F_{1,44}$ = 8.876, P = 0.005, immune $F_{1,44}$ = 0.102, P = 0.751, interaction $F_{1,44}$ = 0.285, P = 0.597). The average mass of puparia produced during this time differed significantly among treatments with both defenses causing lower offspring mass, but again there was no significant interaction between the defenses (Figure 3.6; two-way ANOVA preening $F_{1,962}$ = 46.93, P < 0.0001, immune $F_{1,962}$ = 11.81, P < 0.001, interaction preening*immune $F_{1,962}$ = 2.502, P = 0.11).
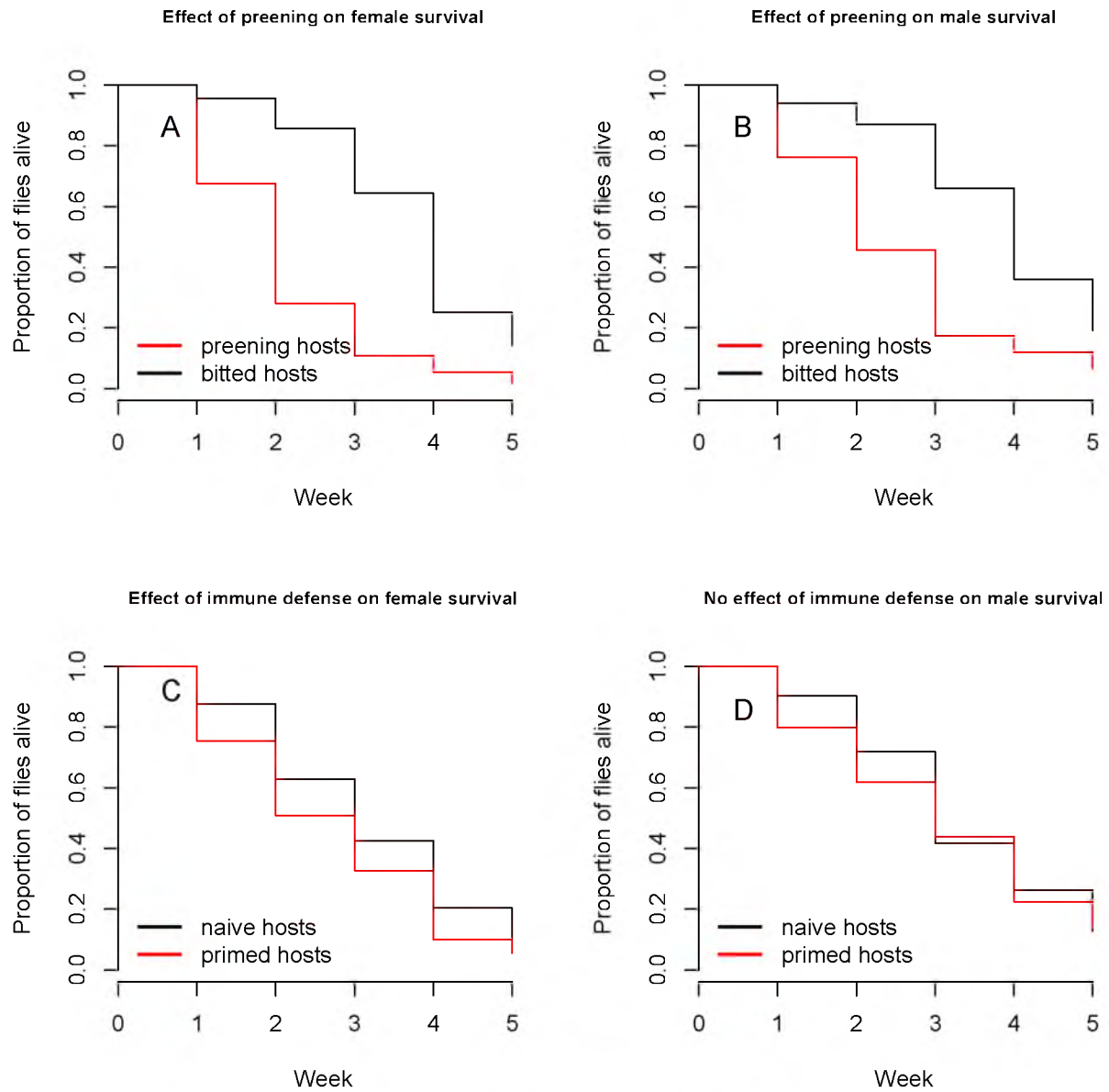
Figure 3.4 – Both female (A) and male (B) flies were significantly affected by preening defense (see text for statistics). However, only female flies (C) were adversely affected by hosts' immune defense over the course of 5 weeks, while males (D) on hosts with and without immunological priming against flies did not differ significantly (see text).
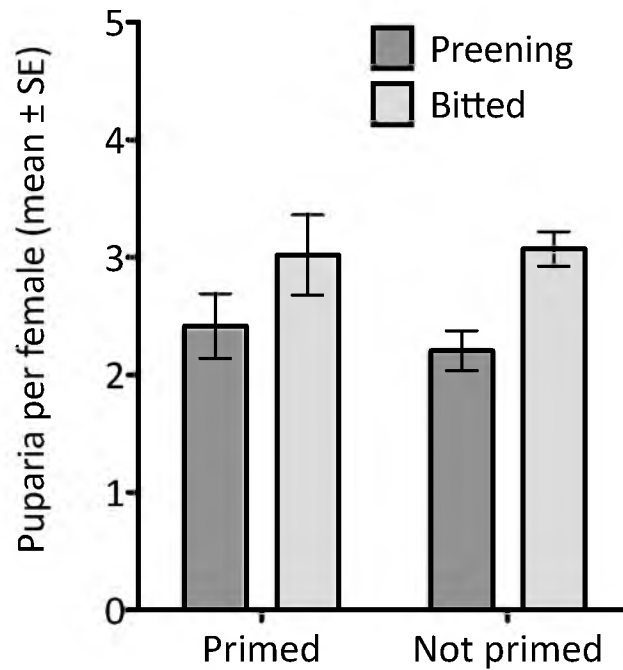
Figure 3.5. Preening lowered female fecundity, but there was no impact of immune response (see text for statistics).

The impact of host defense treatment on *H. columbae* was analyzed using two-way ANOVAs with preening and immune response as factors. The results of these analyses are presented in Table 3.1. Parasite intensity in donor pigeons was correlated with the peak parasite intensity in experimental birds, such that flies fed on more heavily infected donor pigeons transmitted parasites that reached a greater maximum infection intensity in experimental birds regardless of defense treatment; however, only a portion of the variation in experimental infection intensity was explained by donor infection intensity (r-squared = 0.1384, P = 0.009, t = 2.719, $F_{1,46} = 7.392$).
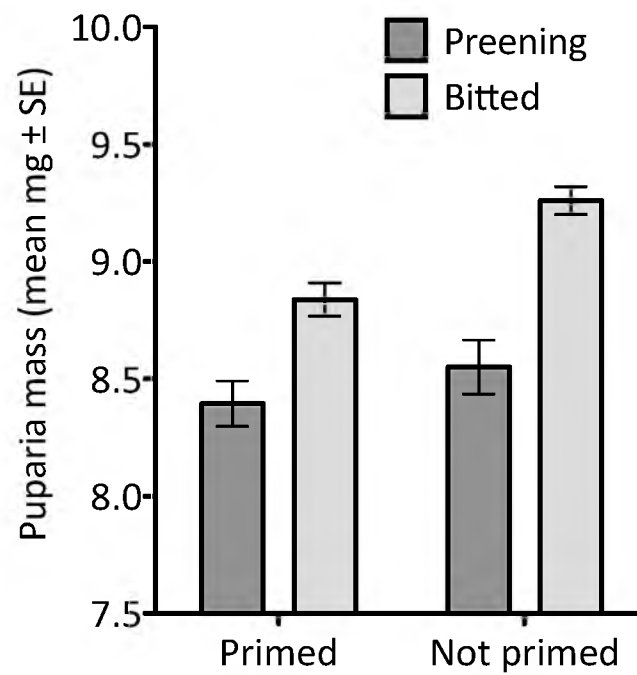
Figure 3.6. Preening and immune defense each lowered mean puparium mass significantly (see text for statistics).

Table 3.1. No influence of host anti-vector defenses on malaria parasite infection dynamics. Non-normally distributed data were rank-transformed within each replicate to normalize them and rankings compared using two-way ANOVAs.

Measure	Preening Defense	Immune Defense	Interaction	Data Transformation	Analysis
Prevalence	P = 1.00		NA	None	Fisher's Exact Test
Peak Infection Intensity	P = 0.134, $F_{1,44}$ = 2.33	P = 1.00, $F_{1,44}$ = 0.0	P= 0.614, $F_{1,44}$ = 0.259	Ranked	2-way ANOVA
Prepatent period	P = 0.214, $F_{1,43}$ = 1.593	P = 0.715, $F_{1,43}$ = 0.136	P = 0.827, $F_{1,43}$ = 0.048	None	2-way ANOVA
Total Parasites Over the Course of Infection	P = 0.319, $F_{1,44}$ = 1.014	P = 0.802, $F_{1,44}$ = 0.063	P = 0.454, $F_{1,44}$ = 0.571	Ranked	2-way ANOVA
Rate of Increase to Peak	P = 0.079, $F_{1,44}$ = 3.237	P = 0.610, $F_{1,44}$ = 0.264	P = 0.798, $F_{1,44}$ = 0.066	Ranked	2-way ANOVA
Rate of Clearance from Peak	P = 0.135, $F_{1,44}$ = 2.343	P = 0.801, $F_{1,44}$ = 0.065	P = 0.801, $F_{1,44}$ = 0.065	Ranked	2-way ANOVA
% Hosts that Cleared the Infection	P = 1.00		NA	None	Fisher's Exact Test

3.5 Discussion

Pigeon host defenses against hippoboscid flies were effective. The behavioral defense of preening against flies was very efficient in killing these vectors of avian malaria parasites, as previously shown (Waite et al. 2012a). Pigeon immune defenses were also effective against the flies, measured in this case by production of specific anti-*P. canariensis* IgY in immune "primed" birds. The mechanism of how the pigeon immune defense works against these mobile ectoparasites is not yet known, nor have molecules in hippoboscid fly saliva been characterized (Sachs and Malaney 2002, Mans 2011). Other studies in model systems suggest potential mechanisms of how vertebrate anti-arthropod immune responses may reduce arthropod feeding, reproduction, and survival. However, using model systems has the potential to produce results that would differ from naturally co-evolved interactions (Randolph and Nuttall 1994, Champagne 2004). Here, our work in a natural system reinforces that done in model systems showing host immune defense against ectoparasites or vectors can be effective.

We did not find any interaction between behavioral and immunological defenses against flies. If these defenses acted synergistically, for instance if immune defenses such as hypersensitivity responses increased itching immediately at the site of a bite, then immune defense could conceivably have directed preening behavior with the combination of defenses having a greater effect on the flies than would be predicted by their additive effects. Delayed type hypersensitivity reactions commonly develop in vertebrates through repeated exposure to vector saliva, but are not usually effective against vectors (Wikel 1996, Titus et al. 2006). By itself, this does not rule out the possibility that immune responses could enhance the effectiveness of another defense, such as by directing

behavioral defenses, or increasing the amount of time spent preening (not measured in this work). An alternative prediction, that defenses act antagonistically, for instance if the cost of one defense prohibited investing fully in the second defense; was also not supported. We find that behavioral and immunological defenses of pigeons against flies work additively, with each defense equally effective on its own or in combination.

When we examined the effectiveness of each of these host defenses against male and female flies separately, we found that preening killed both sexes; however, only female flies were affected by the immune defense. This phenomenon may be explained by the fact that female flies take blood meals that are 40% larger than those taken by males on average (Waite et al. 2012b), and consequently females would imbibe larger quantities of immunoglobulins and may also have increased exposure to proteolytic compounds of the pigeon immune system such as those produced by basophils and eosinophils. Additionally, in our experimental design flies were fed on malaria-infected pigeons prior to being placed on birds in the experiment. Previous work has shown that only female fly survival is affected by malaria parasites, while males are unaffected, possibly due to differences in reproductive investment (Barral et al. 2000, Andrade et al. 2005, Waite et al. 2012b). It is conceivable that female flies were more vulnerable than males to pigeon immune defenses because of their greater stress associated with malaria infection. The preening and immune defenses of pigeons each reduced the average fly offspring mass, a measure of the quality of offspring. However, only preening reduced the female puparia number; immune defenses had no effect on offspring number, only quality.

Rapidly feeding insects such as mosquitoes and other biting flies might not be expected to trigger the immune defenses of their hosts; from a population perspective it

would be undesirable for a host to develop antibodies to proteins in insect saliva that are needed for blood feeding (Schoeler and Wikel 2001). In fact, some blood-feeding arthropods transmit molecules along with the saliva that actively suppress vertebrate immune defenses (Edman and Kale 1970, Gillespie et al. 2000). In some cases such immunomodulatory compounds create more favorable conditions for parasite transmission by making the local host environment more favorable at the feeding site (Darbro and Harrington 2007, Schneider and Higgs 2008, Styer et al. 2011).

Alternatively, previous immune experience of the host to an uninfected vector (or vector saliva) in some cases provides protective immunity from pathogens, including those that cause malaria (Trager 1939, Alger et al. 1972, Kamhawi et al. 2000, Donovan et al. 2007, Gomes et al. 2008). Thus, the outcome of host exposure to an infected vector might be influenced by its history of exposure to vector saliva. Uninfected vectors are often in greater abundance in natural populations than infected vectors (Milleron et al. 2004, Titus et al. 2006, Donovan et al. 2007). If pre-exposure to uninfected arthropod saliva is protective, it may be a natural barrier to parasite transmission. There is some evidence for naturally acquired anti-vector immunity being protective in human populations exposed to sandfly-vectored *Leishmania* (Davies and Mazloumi Gavgani 1999). The mechanism of how an immune response to vector saliva reduces or prevents pathogen transmission is not at all clear in most host-vector-parasite systems, but may polarize the immune system cells to a Th1 rather than a Th2 type response (Wikel 1996, Donovan et al. 2007).

In our study, we found no evidence that pre-exposure of pigeons to uninfected hippoboscid flies afforded any protection from malaria when later exposed to populations

of infected flies (Table 3.1). If any such effect was present, we may have been unable to detect it due to the nature of our experimental design. To track fly survival and fecundity, infected fly populations remained on pigeons for 5 weeks. This design allowed birds to be repeatedly exposed to infected flies for all treatment groups; thus any effect of host defense against flies would have had to act very fast or be very large. Any possible differences in parasitemia due to host defenses against vectors, including immune defenses from preexposure to fly bites, may have simply been overwhelmed. The parasite intensity of donor pigeons explained 14% of the intensity in experimental birds regardless of defense treatment, which suggests additional factors, such as parasite genetic determinants of virulence, explain transmission dynamics (Ferguson et al. 2003, Vardo-Zalik and Schall 2009). The role of genetic variation and malaria parasite virulence for both the insect and vertebrate hosts remains to be explored in this system.

Inherent in the experimental design, flies could not move between birds, and thus any potential influence of pigeon defenses on the host preferences of the flies could not be detected in this design. Indeed, now with the knowledge that anti-vector immune responses are effective against such mobile and relatively rapidly feeding hippoboscid flies, it would be very interesting to learn whether flies prefer hosts without prior immune experience. Anecdotal evidence of flies preferring to feed on nestling pigeons (Acton and Knowles 1914, Bishopp 1929, Coatney 1933) suggests this could be the case, but vector preference for host immune defenses (or lack thereof) is a field that has been little explored. It would be interesting to repeat this experiment with flies permitted to move among hosts and choose which host to bite.

3.6 Acknowledgments

3.7 References

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CHAPTER 4

DOES AVIAN MALARIA REDUCE FLEDGING SUCCESS:

AN EXPERIMENTAL TEST OF THE

SELECTION HYPOTHESIS

ORIGINAL PAPER

Does avian malaria reduce fledging success: an experimental test of the selection hypothesis

Sarah A. Knutie · Jessica L. Waite · Dale H. Clayton

Abstract Like many parasites, avian haematozoa are often found at lower infection intensities in older birds than young birds. One explanation, known as the "selection" hypothesis, is that infected young birds die before reaching adulthood, thus removing the highest infection intensities from the host population. We tested this hypothesis in the field by experimentally infecting nestling rock pigeons *(Columba livia)* with the malaria parasite *Haemoproteus columbae*. We compared the condition and fledging success of infected nestlings to that of uninfected controls. There was no significant difference in the body mass, fledging success, age at fledging, or post-fledging survival of experimental versus control birds. These results were unexpected, given that long-term studies of older pigeons have demonstrated chronic effects of *H. columbae*. We conclude that *H. columbae* has little impact on nestling pigeons, even when they are directly infected with the parasite. Our study provides no support for the selection hypothesis that older birds have lower parasite loads because parasites are removed from the population by infected nestlings dying. To our knowledge, this is the first study to test the impact of avian malaria using experimental inoculations under natural conditions.

Keywords *Columba livia* · Pigeon · Fitness · Hippoboscid fly · Host-parasite interaction

Introduction

Parasites influence fundamental aspects of the evolutionary ecology of their hosts, such as population dynamics (Anderson and May 1978; Anderson 1979) and life history evolution (Hochberg et al. 1992). The impact of parasites on host fitness depends partly on the age at which hosts become infected. A common pattern in host-parasite interactions is that

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younger individuals have higher parasite loads than adults (Gregory et al. 1992; Hudson and Dobson 1997). Sol et al. (2003) considered three hypotheses to explain this pattern. The "selection" hypothesis suggests that highly parasitized juvenile hosts die before they reach adulthood, removing large numbers of parasites from the population. The "immunity" hypothesis suggests that the developing immune system of juveniles is not yet capable of killing parasites, while adults are much more effective at reducing parasite intensity. The "vector exposure" hypothesis suggests that adult behavior reduces their exposure to infected vectors, and thus parasites, compared to juveniles.

Sol et al. (2003) evaluated these hypotheses using data from a study of feral rock pigeons *(Columba livia)* infected with malaria parasites *(Haemoproteus columbae)* vectored by pigeon louse flies (Hippoboscoidea: *Pseudolynchia canariensis)*. The authors rejected the vector exposure hypothesis because they found that adult pigeons (>6 months old) are not, in fact, exposed to fewer vectors than juvenile pigeons (Sol et al. 2000). Although the authors reported higher rates of juvenile mortality (61 %) compared to adult mortality (33 %), consistent with the selection hypothesis, selection in their study was not strong enough to explain the lower number of parasites observed in adult birds. The youngest birds in Sol et al.'s study had already fledged from the nest; however, the greatest impact of *H. columbae* on pigeons may occur while birds are still in the nest. We conducted a study to test the impact of *H. columbae* on the condition and fledging success of younger, nestling rock pigeons. We used an experimental approach in which we compared nestlings injected with *H. columbae* to control birds not injected with the parasite.

At least 200 species of *Haemoproteus* are known to infect birds worldwide (Martinsen et al. 2008). Perez-Tris et al. (2005) classified *Haemoproteus* as an avian malaria parasite because members of the genus were nested phylogenetically within the genus *Plasmodium*. *H. columbae* is a parasite of pigeons and doves that uses blood-feeding pigeon flies as vectors (Valkiūnas 2005). The parasite enters a feeding fly and reproduces in its midgut, where *H. columbae* oocysts attach to the gut wall. Once mature, the oocysts burst and release infective sporozoites that migrate from the fly's gut into its salivary glands. The fly then injects these sporozoites into a pigeon when it feeds. *H. columbae* reproduces asexually in the lungs of the pigeon, then invades and matures in the red blood cells (Ahmed and Mohammed 1978).

Haemoproteus species can have several negative effects on host fitness. These effects include reductions in host body condition (Merino et al. 2000), lower reproductive success (Marzal et al. 2004; Tomas et al. 2007), and even death (Atkinson and Forrester 1988; Sol et al. 2003). Studies of the impact of malaria on juvenile birds have consisted of observational studies in the field (Sol et al. 2003), and experimental studies using captive birds (Yorinks and Atkinson 2000; Garvin et al. 2003). The goal of our study was to use an experimental approach under field conditions. We infected nestling birds with malaria parasites to test the impact on body mass, fledging success, age at fledging, and post-fledging survival of experimental versus control birds. Studies with captive birds suggest that the most pathogenic phase of the *Haemoproteus* life cycle occurs when parasites enter red blood cells to mature (Atkinson and Forrester 1988; Atkinson and van Riper 1991). In the case of *H. columbae* this takes place about 24–37 days after infection (Ahmed and Mohammed 1978). Since pigeons fledge at about 32 days of age, it is not possible to be sure that fledglings are infected with malaria parasites, short of experimentally infecting them. Experimental manipulation is the most powerful approach for testing the impact of parasites on hosts in any case (McCallum and Dobson 1995). To our knowledge, this is the first study to test the impact of avian malaria parasites using experimental inoculation under natural conditions.

Materials and methods

We experimentally manipulated *H. columbae* in nestling rock pigeons. The study took place August–November 2009 under a highway overpass in Draper, Utah, USA (40°31′36″N, 111°53′28″W). We visited the field site every 2–3 days throughout the study period. Nestlings were weighed at each visit to the nearest 1.0 g with a pesola scale. Our experiment was restricted to nests with two nestlings, the normal number for rock pigeons. Nests were sequentially assigned to one of three treatment groups: experimental (n = 12 nests), control (n = 13), or background (n = 12). When nestlings were 4–7 days old (50–150 g), those at experimental nests were injected with a suspension of *P. canariensis* flies infected with *H. columbae* (Ahmed and Mohammed 1978). We created the infected fly suspension by feeding flies (bred from wild stock) on heavily infected captive birds. Following 10–12 days on a bird, flies were placed in vials and taken to the field site, where batches of ten live flies were macerated in 1,000 µL of phosphate buffered saline for 3 min. Experimental nestlings were injected intraperitoneally with 500 µL of the infected fly suspension using a 0.5 cc syringe. Control birds were injected with 500 µL of another suspension made using uninfected flies. Background birds were handled but not injected.

Prior to the field experiment, we conducted a test of the inoculation method using 27 wild trapped, captive rock pigeons. After blocking by capture date and site, 13 randomly chosen birds were injected with a suspension of infected flies, as described above. Fourteen control birds were injected with a suspension of uninfected flies. At 25, 35, and 42 days post injection, blood samples were taken from all birds and smears were prepared for examination. Each smear was carefully examined under oil immersion at 1,000 × for 10 min; if parasites were detected, then the number of parasites was quantified in 25 microscope fields per bird. All 13 experimental birds were infected with *H. columbae*, while none of the 14 control birds was infected.

When nestlings were approximately 10 days old they were fitted with a numbered aluminum band and three plastic color bands. To score fledging success we observed and identified birds after they left the nest on the basis of their color band combinations. We conducted a thorough census of all birds at the bridge during each visit to the field site. We also searched for banded birds at other bridges within 8 km of the study site in order to determine whether newly fledged birds were dispersing from the natal site.

We continued to monitor birds at the bridge for 50 days post injection (ca. 25 days post fledging) because peak parasitemia can be delayed for this long after injection (extrapolated from Ahmed and Mohammed 1978). To confirm experimental infections, we examined the blood of birds after they fledged. We used walk-in traps to capture pigeons from 30–50 days post injection. Blood samples were taken and birds immediately released. Blood smears were prepared and examined back in the lab.

Data were analyzed using Prism® v.5.0b (GraphPad Software, Inc.). Power analyses were conducted in G*Power 3 with an error probability set at 0.05 (Buchner et al. 1997). Where necessary, data were log transformed for normalization. To avoid pseudoreplication (Hurlbert 1984) we averaged values for nestlings within each nest. We used one-way ANOVAs to compare parasite abundance and host age and mass at fledging among treatments. A repeated-measures ANOVA was used to compare the number of birds per nest at hatching, fledging, and 1, 2, and 3 weeks post-fledging.

Results

Three times as many experimental birds were infected as control or background birds (Fig. 1a); the three groups also differed in parasite abundance (Fig. 1b; ANOVA $F_{2,17} = 4.25$, $P < 0.05$). Dunnett's post hoc comparisons confirmed that experimental birds had significantly more parasites than controls ($P < 0.05$), while control and background birds did not differ significantly ($P > 0.05$).

There was no significant difference in the age of birds at fledging, nor body mass prior to fledging (see Table 1). There was no significant difference in the proportion of nests that fledged at least one offspring ($\chi^2 = 0.005$, $P = 0.99$). There was also no significant effect of treatment on the mean number of birds fledged per nest, nor the number of birds observed after fledging (Fig. 2; repeated measures ANOVA, treatment $F_{2,34} = 0.64$, $P = 0.53$). There was a significant effect of time (Fig. 2; time, $F_{4,136} = 43.32$, $P < 0.0001$), but no significant interaction between time and treatment (time*treatment, $F_{8,136} = 0.49$, $P = 0.86$).

We reanalyzed the data after excluding naturally infected control and background birds, as well as experimental birds for which we could not confirm infection. We still found no significant difference in age at fledging ($F_{2,31} = 0.53$, $P = 0.60$) mass at fledging ($F_{2,31} = 1.01$, $P = 0.38$), or the proportion of nests that fledged at least one offspring ($\chi^2 = 0.01$, $P = 0.99$).

Our experiment had considerable power (1.0) to detect the level of juvenile mortality (61 %) reported by Sol et al. (2003), we had power of 0.8 to detect mortality of at least 30 % (effect size of f = 0.55).

Discussion

Our goal was to experimentally test the "selection" hypothesis. This hypothesis, reviewed by Gregory et al. (1992), states that lower parasite loads of adults, compared to juveniles, are the result of heavily infected juveniles dying before adulthood, removing parasites from the population. Previous tests of this hypothesis involving avian malaria have focused on juvenile (fledged) birds and relied on observational data (Sol et al. 2003;
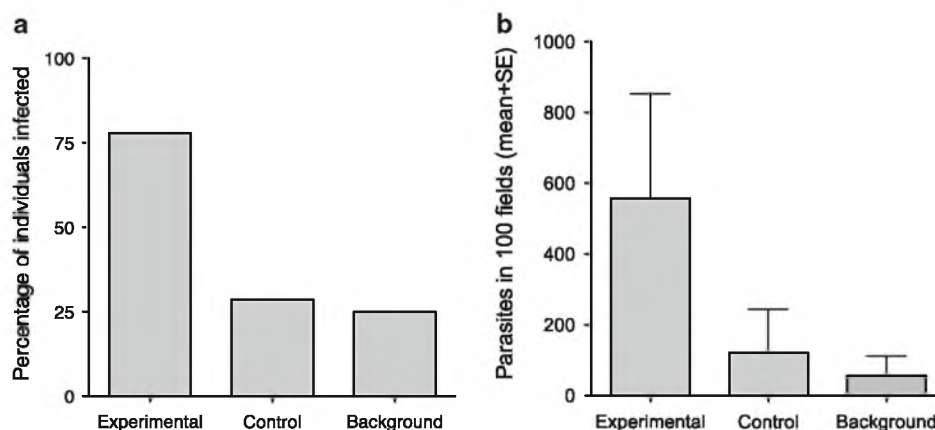
Fig. 1 Prevalence (a) and mean abundance (+SE) (b) of malaria parasites 30–50 days after treatment

Table 1 Age of birds at fledging and body mass prior to fledging. Values are grand means (±SE) of the mean value per nest

	Experimental	Control	Background	Test statistic	P
Age in days	32.3 ± 0.5	31.8 ± 0.6	32.3 ± 0.6	$F = 0.25$	0.78
(Number of nests)	(11)	(12)	(11)		
Mass in grams	298 ± 14.9	311.3 ± 12.5	313.1 ± 11.4	$F = 0.35$	0.71
(Number of nests)	(11)	(12)	(11)		

Fig. 2 Mean (±SE) offspring observed per nest. The mean (±SE) number of offspring fledged per nest did not differ significantly among treatments. Points are slightly offset for clarity
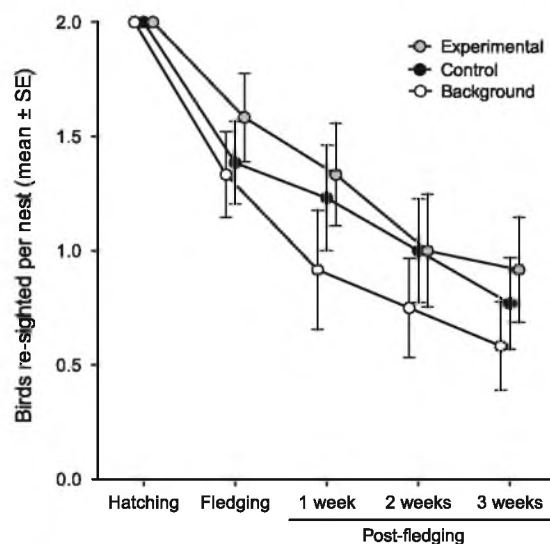
van Oers et al. 2010). These studies provided some support for the selection hypothesis, but the intensity of selection measured could not fully explain differences in juvenile and adult parasite loads. It was conceivable, therefore, that the greatest impact of *H. columbae* on pigeons takes place while they are still in the nest.

Our results provided no support for the selection hypothesis because there was no impact of malaria on any of the components of host fitness we measured. Specifically, there was no significant difference in the body mass, fledging success, age at fledging, or post-fledging survival of experimental versus control birds. We are confident that our measures of post-fledging survival were accurate because none of the birds from our study were observed at other bridges (see methods). Young pigeons do not normally disperse until 3 months of age, in any case (Johnston and Janiga 1995).

The results of our study were unexpected, given that Sol et al.'s longer-term study demonstrated that *H. columbae* has a significant negative impact on pigeon fitness. The fact that malaria had no detectable impact on fledging success in our study was not due to unusually low rates of fledging in both experimental and control birds. Fledging success was 73 % (Fig. 2), similar to that in other studies of feral pigeons [reviewed by Johnston and Janiga (1995), Table 18.4 (values adjusted for hatching rates)]. Similarly, the fact that malaria had no detectable impact on fledging was not due to methodological problems with the creation of experimental infections. The malaria parasite levels in our study were comparable to those observed in other studies of naturally infected pigeons (Kartman 1949;

Klei and DeGuisti 1975; Paperna and Smallridge 2002). However, *H. columbae* may affect hosts only at levels higher than what we observed (Earle et al. 1993; Paperna and Smallridge 2002). For example, the *H. columbae* levels in Sol et al.'s (2003) study were among the highest ever recorded for feral rock pigeons.

Another factor that could conceivably contribute to why the birds in our study did not appear to be affected by *H. columbae*, compared to the reduction in survival shown for older birds by Sol et al. (2003), is that nestling pigeons could have higher tolerance to parasites than older birds. Nestlings are fed a rich diet of crop milk by both parents. The milk, which consists of the sloughed lining of the parents' crop, is very high in fat and protein (Johnston and Janiga 1995). It would be interesting to test the impact of *H. columbae* on nestlings fed a less nutritious diet.

A few control and background birds were naturally infected with *H. columbae*. However, infection levels were still significantly higher in the experimental group than the control or background groups. Even after excluding the naturally infected birds, we did not find that malaria parasites affected fledging age or mass, or fledging success.

Since *H. columbae* had no apparent effect on nestling rock pigeons, our study does not provide support for the "selection hypothesis". Sol et al. (2003) reported results that were consistent with selection hypothesis; however, selection in their study was not strong enough to explain the differences in parasitemia they observed between juvenile and adult pigeons. Because Sol et al. (2000, 2003) reported data ruling out the "vector exposure" hypothesis, they suggested a combination of the selection and immunity hypotheses may explain the fact that juvenile birds have higher parasitemia than adult birds. Our data provide no reason to disagree with this assessment.

To our knowledge, this is the first study to test the impact of avian malaria parasites using experimental inoculation under natural conditions. This approach has several advantages. First, like many malaria parasites, *H. columbae* takes several weeks to appear in the peripheral blood after the host is infected. This fact makes early infections difficult to detect without more invasive methods, such as collection of organ tissues (Valkiūnas 2005; Cosgrove et al. 2006). Experimental infections get around this problem. Second, inoculating hosts with parasites has the strong advantage of controlling for factors that could lead to spurious negative correlations between parasite load and host fitness (Hawlena et al. 2006; Blanchet et al. 2009). The greatest limitation of our study is that the modest sample sizes limit our ability to detect relatively small effect of malaria parasites on birds. For example, to detect a 10 % reduction in juvenile survival with a power of 0.8 would require a sample of 93 nests per treatment for a total of 279 nests. A study of this magnitude may be feasible in the future using feral Rock Pigeons and *H. columbae*.

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CHAPTER 5

SEX-SPECIFIC EFFECTS OF AN AVIAN MALARIA

PARASITE ON AN INSECT VECTOR:

SUPPORT FOR THE RESOURCE

LIMITATION HYPOTHESIS

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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
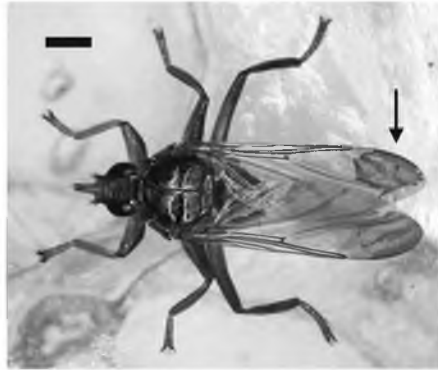
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FIG. 1. Pigeon fly (*Pseudolynchia canariensis*) with color marks applied on each wing tip (arrow) for individual identification. The scale bar in the upper left corner is 1 mm.

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 trans-ovarially, 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 $\pm$ 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
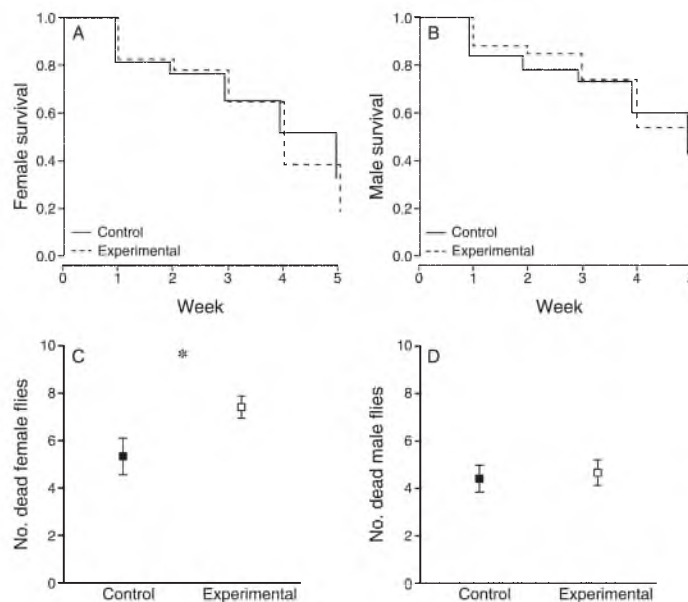
Fig. 2. (A, B) Proportion of flies surviving over time, by sex. Survival on experimental (infected) birds was less than that on control (uninfected) birds for female flies (A), but not male flies (B); see text. Cumulative (C) female and (D) male fly mortality (mean ± SE) at the end of the five-week experiment. 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

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

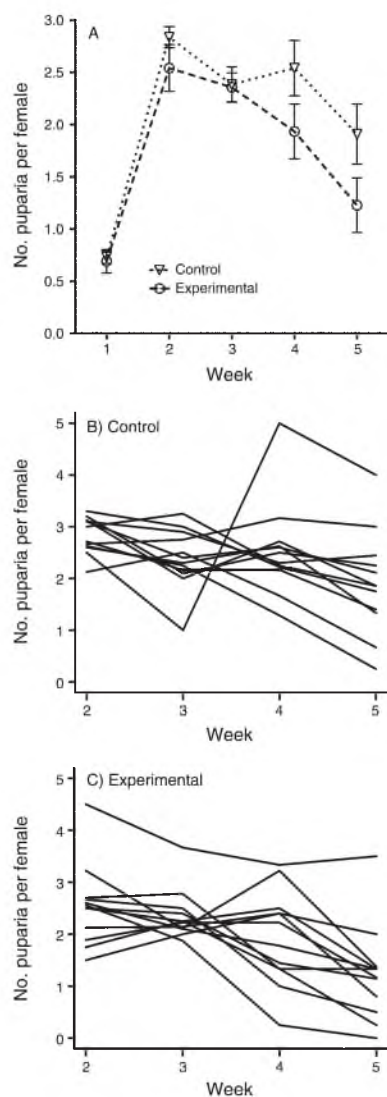FIG. 3. Comparative reproductive success of flies on experimental (infected) and control (uninfected) birds: (A) Number of puparia deposited by female flies (mean ± SE). pooled for the 12 birds in each treatment. For individual birds, the mean numbers of puparia per week are shown for (B) control and (C) experimental birds. Flies on experimental birds produced significantly fewer puparia than flies on control birds.

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
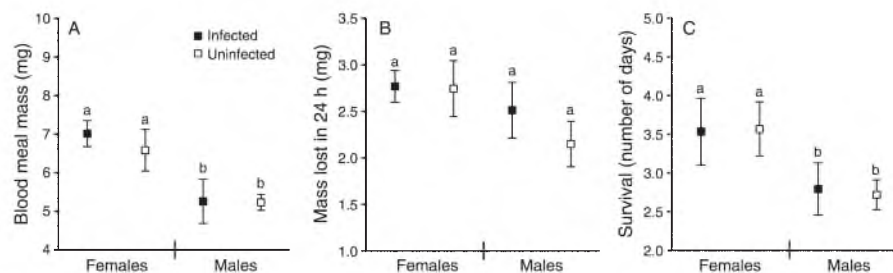

FIG. 4. Feeding experiment results (mean ± SE): (A) blood meal size of flies on infected and uninfected birds; (B) amount of blood digested (measured as mass loss) over 24 hours; (C) number of days flies survived after being removed from hosts. Different lowercase letters indicate significant differences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

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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CHAPTER 6

CONCLUSION

The broader importance of the experiments described here lies in expanding our knowledge of and testing ideas about the ecology and evolution of vector-borne parasites. The outcomes of specific interactions within a host-vector-parasite system can inform our understanding of the forces that shape the evolution of disease dynamics. Conventional wisdom states that a parasite should not harm its vector, but should instead be more virulent to its main host (Ewald 1994). In malaria parasite systems, the insect host is typically thought of as the vector, and the vertebrate host as the main host. In this system of Rock Pigeon, hippoboscid fly, and malaria parasite, and the experiments described here, these predictions are not upheld.

I found the malaria parasite reduces the survival of its female vectors (but does not harm males), and did not find any effect of the parasite on the main host, at least in very young, nestling pigeons. The terms "vector" and "main host" seem inadequate to describe this pathogen system, and perhaps others. In parasitology, the definitive host is the one in which the parasite undergoes sexual reproduction; the importance of this part of the parasite's life cycle suggests the definitive host could be considered the "main" host. For all malaria parasites, sexual reproduction takes place within the insect host. A vector is often defined as any agent that transmits pathogens between hosts. In this case, perhaps pigeons could be thought of as "vectors" given that they are likely to fly further than the

hippoboscid flies and could be potentially more important in moving parasites longer distances. The fly is necessary in this system to transmit parasites to new pigeons, but the opposite could be argued as well – that pigeon movement is necessary to transmit parasites to new flies.

The pigeon-fly-malaria system presents a unique opportunity for examining the ecology of a parasite that is transmitted by both fly sexes. Across the diversity of insect vectors it is rare that male flies transmit parasites (Otranto et al. 2008); it would be interesting to further explore the dynamics of disease systems with multiple vectors, whether these be both sexes or multiple species. *Haemoproteus columbae* has no significant effect on male flies, yet it decreases female fly survival and reproduction. The effects act later in life and are most pronounced when female *P. canariensis* are older and/or exposed to the parasites for a longer period of time. The relative contribution of these last two factors (age and duration of exposure) in reducing fly fitness could be determined experimentally by moving *P. canariensis* females of various ages to pigeons with and without malaria. Additionally, as discussed in Chapter 5, the relative effect of repeated pregnancies versus normal female physiology (taking larger meals for instance) on the pathological effects of *H. columbae* could also be determined by further experimentation. If the female-specific effect is largely a result of higher reproductive costs than males, one prediction is that virgin female flies would have survival equivalent to, or even exceeding that of male flies. Some female hippoboscids typically live longer than males (Bequaert 1953).

Currently, few non-human malaria model study systems have been explored to more broadly understand the interactions of malaria parasites with their hosts and vectors.

Those that do exist may use unnatural host-vector-parasite associations, or do not provide the opportunity to study the complete parasite life cycle under laboratory conditions. Expanding work in atypical or nontraditional natural systems has the potential to reshape how we think about evolutionary theory, particularly how we might expect parasites to co-evolve with their vertebrate as well as arthropod hosts.

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APPENDIX A

PREVALENCE AND ABUNDANCE OF *HAEMOPROTEUS*

COLUMBAE IN THE SALT LAKE CITY, UT

VALLEY BY LOCATION AND SEASON

A.1 Introduction

Tracking seasonal and spatial variability of parasites in wild host populations can be incredibly useful for understanding the forces that drive parasite ecology, transmission, and ultimately evolution (Altizer et al. 2006). Surveys across many different locations can be particularly valuable for addressing these objectives. Here I present the results of a survey of *Haemoproteus columbae* in feral Rock Pigeon *(Columba livia)* populations in Salt Lake City, UT. At least 20 published surveys have documented the prevalence of *H. columbae* in other pigeon populations worldwide, allowing for comparisons between pigeon populations across the globe (Table A.1). To my knowledge, this is the first such study done in the Southwest United States, where *H. columbae* prevalence was previously unknown. The locations of the previous surveys of *H. columbae* prevalence are depicted here on a world map (Figure A.1).

Local *H. columbae* prevalence can be highly variable, even between relatively close geographic locales e.g. (Giovannoni 1946, Sol et al. 2000), perhaps due to the patchy distribution of the hippoboscid flies *(Pseudolynchia canariensis)* which transmit the malaria parasites (Sol et al. 2000). Although many previous surveys (Table A.1) have examined both *H. columbae* prevalence and prevalence of its vector simultaneously, quantitative data on fly distributions in the southwest USA (including Salt Lake City, UT) were not available. I therefore set out to obtain such data.

Table A.1. Summary of global studies of *Haemoproteus columbae* prevalence in feral Rock Pigeon populations. Prevalence has been rounded to the nearest percentage.

Study Site	No. *C. livia*	Mean Prevalence	Dates Sampled	Reference	Fig. A.1 ID
Honolulu, HI, USA	101	82%	JAN - FEB 1948	Kartman 1949	A
Salt Lake City, UT, USA	122	66%	NOV 2006 –JUN 2010	Waite, current study	B
Galapagos Islands, Ecuador	28	0%	JUL 2001 – JUN 2002	Padilla et al. 2004	C
Detroit, MI, USA	754	78%	APR 1966 - SEP 1968; MAR 1969 - FEB 1971	Klei 1971, Klei and DeGuisti 1975	D
VA, USA	60	58%	<1962 dates NA	Jochen 1962	E
Hyattsville, MD, USA	30	60%	DEC 1961	Knisley and Herman 1967	F
Lages, Santa Catarina, Brazil	58	67%	APR-AUG 2004	Tietz Marques et al. 2007	G
Uberlandia, Minas Gerais, Brazil	34	100%	2000?	Oliveira et al. 2000	H
Brazil	159	58%	<1946 dates NA	Giovannoni 1946	I
Santa Cruz de Tenerife Island (Canary Islands)	50	82%	JAN-DEC 2001	Foronda et al. 2004	J
Madrid, Spain	118	97%	2006-2007	Vázquez et al. 2010	K
Catalonia, Spain	257	80%	FEB-JUL 1998	Sol et al. 2000	L
Sebele, Gaborone, Botswana	30	75%	NA, ~1999	Mushi et al. 1999	M
Uganda	34	76.5%	OCT 1996-MAR 1997	Dranzoa et al. 1999	N
Mogoro Municipality, Tanzania	200	37%	JAN-MAR 2007	Msoffe et al. 2010	O
Tel Aviv, Israel	97	76%	NOV 1998 - OCT 1999	Paperna and Smallridge 2002	P
Birjand, South Khoasan, Iran	102	47%	OCT 2008 – SEP 2009	Radfar et al. 2011	Q
Dehli, India	75	100%	1946-1950	Singh et al. 1951	R
Garia, West Bengal, India	45	100%	NOV 1986-OCT 1987	Mandal 1991	S
Singapore	79	95%	MAR-JUN 1998	Paperna and Smallridge 2002	T

Figure A.1. Geographic locations where feral Rock Pigeons *(Columba livia)* have been surveyed for *Haemoproteus columbae* prevalence. Letters designate locations listed in Table A.1. Locations are labeled alphabetically from east to west on the map. Data from the current survey in Salt Lake City, UT USA (B) fills a significant geographic gap.

A.2 Methods

A.2.1 Pigeon samples

Blood was sampled from 122 pigeons trapped in the Salt Lake City, UT valley. The location and date of capture was recorded for each pigeon. Sampling was opportunistic, and thus date and location of sampling were often highly correlated for a single site. In some cases, pigeons captured at two sites along the Interstate-15 corridor (locations 4500 S and 5300 S) were trapped at the same date and birds were not kept separate, thus data for these two sites were combined, and the exact origin of a bird could not be determined. The time that pigeons spent in captivity before their blood was sampled for *H. columbae* was recorded, and only birds sampled within one month of capture were included in this study to estimate *H. columbae* prevalence. For further details on the timing of the life cycle of *H. columbae* see (Ahmed and Mohammed 1978, Knutie et al. 2012, Waite et al. 2012).

A.2.2 Blood samples

Blood was collected by brachial venipuncture into a 70 µL heparinized capillary tube. Two droplets were used to make duplicate blood smears to examine the blood for parasites. Small drops of blood were put onto duplicate frosted-end glass microscope slides and thin blood smears were made by sliding one slide over the other with slight pressure from the top slide held at a 45° angle. Thin blood smears were dipped in methanol for 1 minute and then stained with a dilute solution of Baker brand Giemsa stain and buffered distilled water (1:10, pH 7.0) for 50 minutes, rinsed in buffer and allowed to dry before examination. Smears were examined under microscopy at 1000x using an oil immersion lens. Slides were carefully scanned for 20 minutes to determine

the infection status (positive or negative) of each pigeon sampled. For most infections (104/122) parasite intensity was roughly estimated. Most of the samples that were not estimated were taken from birds in the "Avenues" neighborhood of Salt Lake City, UT (N = 18). Parasite intensity was estimated categorically using the following criteria for the number of parasites seen at 1000x magnification:

- "Very Heavy" (4): greater than one parasite seen in each microscopy field on average

- "Heavy" (3): about one parasite seen in each field

- "Medium" (2): about one parasite seen in every other field

- "Light" or "Very Light" (1): one parasite every 5 or fewer fields.

- "Zero": no parasites observed.

A.3 Results

I found no significant seasonal or spatial variation in *H. columbae* infection prevalence across pigeon populations in the Salt Lake City, UT valley. Table A.2 summarizes sampling efforts at each location over time.

While I found a difference in overall prevalence across months (Figure A.2, Kruskal-Wallis statistic = 11.78, P = 0.02), there was no difference in *H. columbae* prevalence between any pairwise set of months (Dunn's multiple comparison tests, all P >0.05). Intensity did not differ significantly by month (Figure A.3, Kruskal-Wallis statistic = 2.47, P = 0.65). *H. columbae* prevalence or intensity of infection did not differ due to sampling location (Prevalence, Figure A.4, Kruskal-Wallis statistic = 10.05, P = 0.07; Intensity, Figure A.5, Kruskal-Wallis statistic = 6.45, P = 0.26).

Table A.2. Locations and dates where pigeons were sampled in the Salt Lake City, UT area. The number of pigeons that were sampled at each site are provided.

Sampling Month	Month					
	Jan	Mar	June	Aug	Nov	Dec
Sampling Year(s)	2008	2008	2010	2008	2006, 2007	2007, 2008
No. Pigeons	13	20	36	1	22	30
Location(s)	Univ. of Utah	Avenues	4500S I-15	45/5300S I-15	45/5300S I-15, 5300S I-15	5300S I-15, 7200S I-15, Univ. of Utah
GPS coordinates, Latitude-Longitude	Univ. of Utah: 40.763349,-111.849446	Avenues: 40.771694,-111.87642	4500S I-15: 40.674487,-111.901374		5300S I-15: 40.654857,-111.901245	7200S I-15: 40.620924,-111.903992
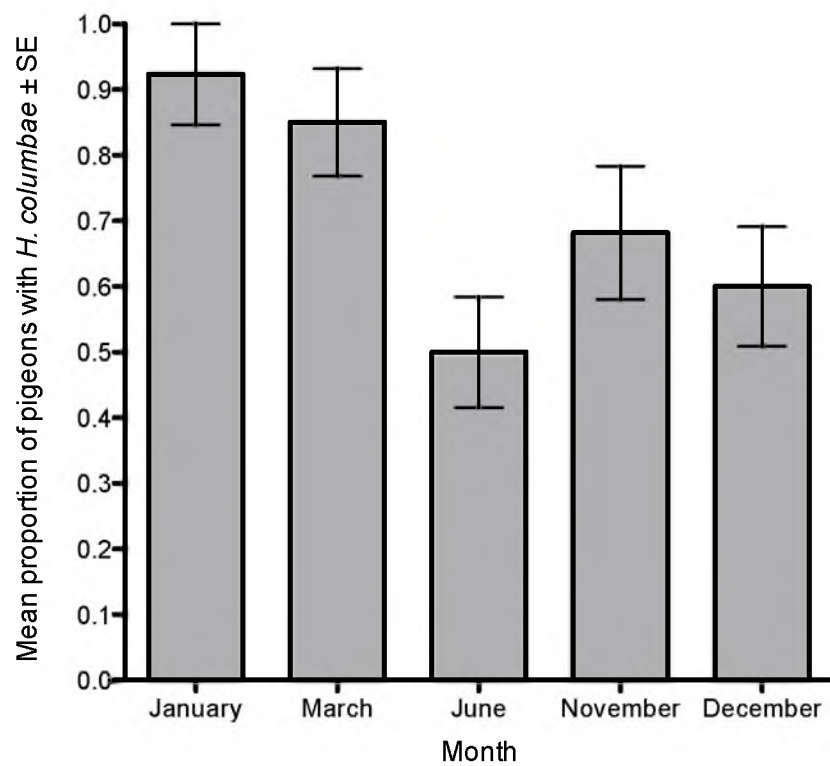
Figure A.2. The proportion of feral Rock Pigeons infected with *H. columbae* was significantly different among months, although no two months were significantly different from one other in pairwise comparisons.
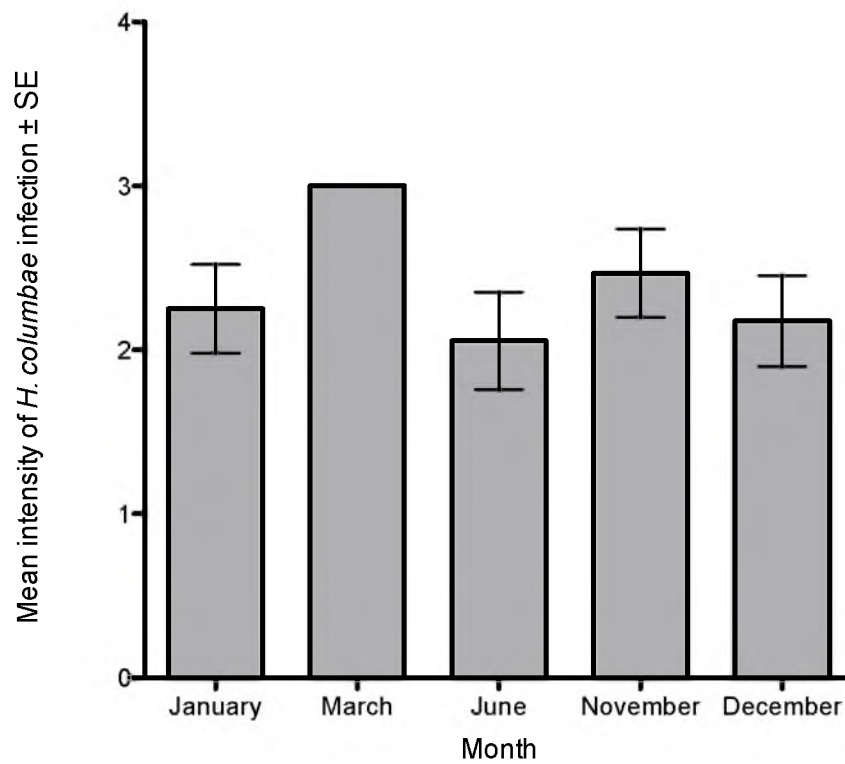
Figure A.3. There was no difference in the estimated intensity of *H. columbae* infection in infected feral Rock Pigeons based on the month they were sampled.
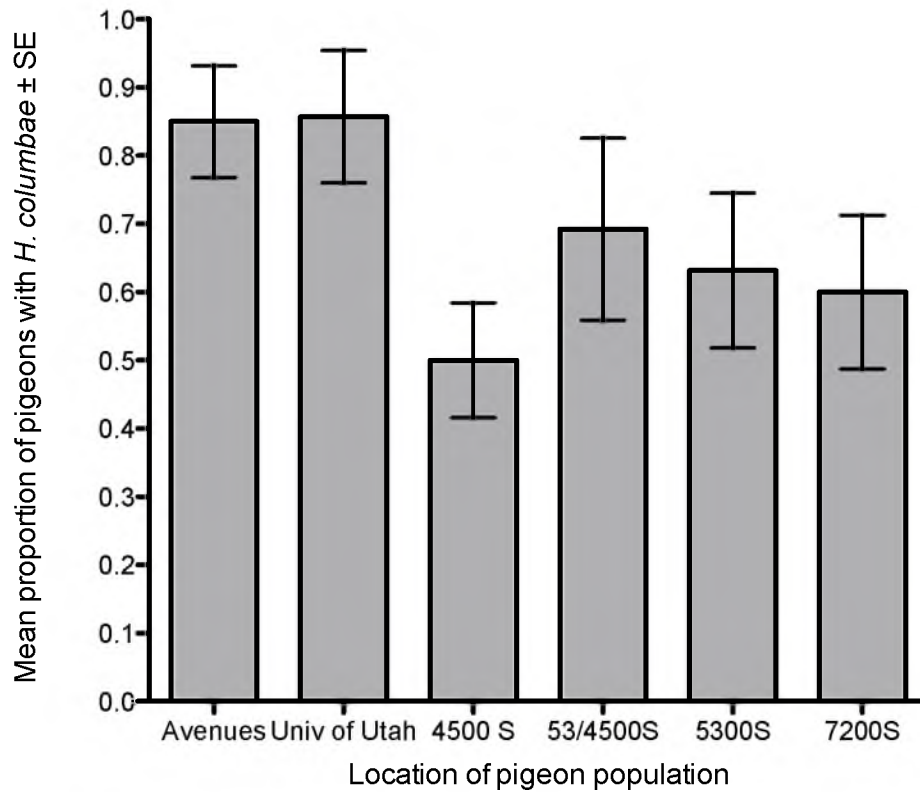
Figure A.4. The proportion of feral Rock Pigeons that were infected with *H. columbae* did not differ by location of the pigeon population in the Salt Lake City, UT valley. Locations are organized from North to South.
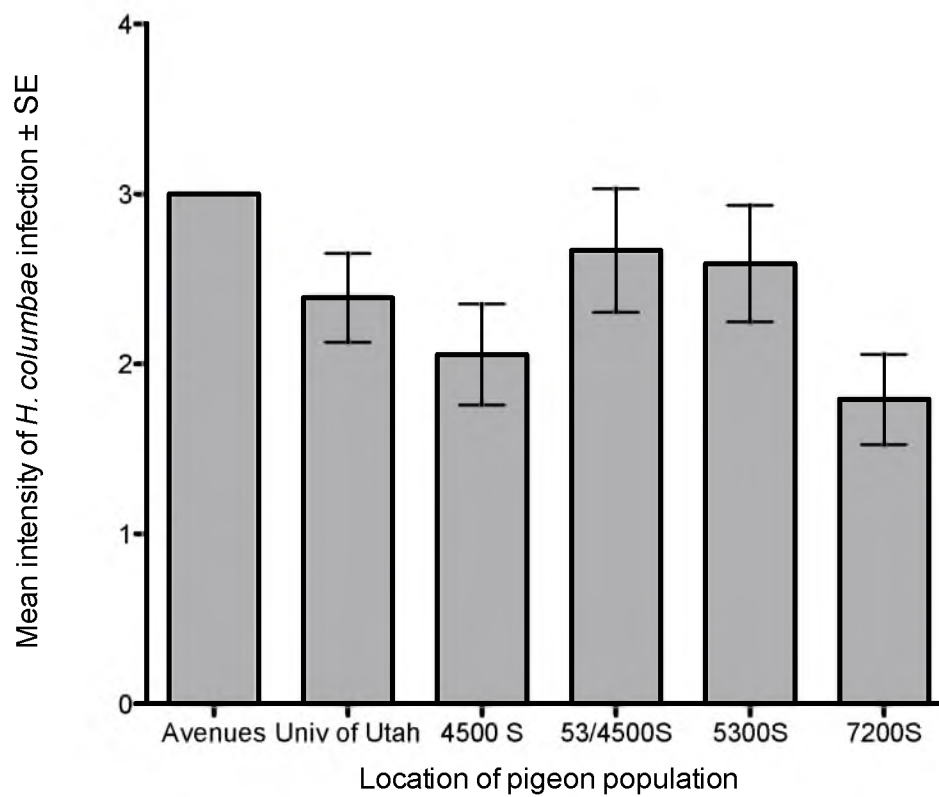
Figure A.5. Estimated intensity of *H. columbae* infection in feral Rock Pigeons did not differ among locations in the Salt Lake City, UT valley. Locations are arranged from North to South.

A.4 Discussion

Given the opportunistic nature of blood sampling in this study, comparisons of *H. columbae* prevalence across locations, dates, and seasons in pigeon populations in Salt Lake City, UT are difficult to interpret. Perhaps the most useful aspect of this study is formal documentation of an average 66% prevalence of *H. columbae* in the area, and that seasonal variation in prevalence may exist, similar to other studies of *H. columbae* (Klei and DeGuisti 1975) and other *Haemoproteus spp.* parasites (Schrader et al. 2003). I did not find any relationship between sampling location and parasite prevalence, as has been found in other studies of *H. columbae* (Giovannoni 1946, Sol et al. 2000). This result could suggest that my sampling sites are close enough together that pigeon populations are mixing. Alternatively, conditions influencing flies across the sampling area could simply be equivalent, with similar populations of flies present and relatively equal parasite transmission. Finally, the lack of independence between sampling date and location in our opportunistic sampling design could have masked any real patterns.

Given the frequent surveys for *H. columbae* in global pigeon populations, this system could be an excellent candidate for modeling efforts that could help predict parasite transmission. Although some modeling efforts have been made (Beaudoin et al. 1971, Gabaldon and Ulloa 1980), there is certainly room for expansion. Previous studies have shown seasonal (Klei and DeGuisti 1975) and geographic (Sol et al. 2000) variation in prevalence. One study also compared prevalence between two locations (Singapore and Israel) (Paperna and Smallridge 2002) and while they found no difference in hippoboscid fly prevalence between the locations, the prevalence and parasitemia of *H. columbae* was higher in Singapore than Israel. Work like that of Paperna and Smallridge (2002)

conducted across additional locations might yield more generalized conclusions and perhaps better define the role of the environment in malaria transmission dynamics.

It is interesting to note that the only location where *H. columbae* was not detected in surveys for this parasite is the Galapagos Islands, Galapagos, Ecuador. It is possible that *H. columbae* is not present on the islands, especially if the vector, *Pseudolynchia canariensis*, was also absent. Alternatively, *H. columbae* may have been present but was not found because the sampling period was short and sampling of Rock Pigeons was limited (Padilla et al. 2004). Since Padilla et al.'s study, *C. livia* has been eradicated from the Galapagos and so *P. canariensis* and *H. columbae* should not be present (Clayton *pers com.*).

No clear pattern of distribution emerges from currently published surveys of *H. columbae* prevalence; however, since the vector *P. canariensis* has climate limitations for overwintering (Klei and DeGiusti 1975), there is a latitudinal range outside which *H. columbae* would not be expected. Because negative results are often harder to publish (Fanelli 2011), surveys of Rock Pigeon populations where *H. columbae* was not found may not be as readily available. Surveys beyond the boundaries of the latitudes already examined (Figure A.1), in places such as Canada, Russia, and southern Argentina would be informative.

A.5 Acknowledgements

A.6 References

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APPENDIX B

MOLECULAR GENETIC TOOLS DEVELOPED

FOR *HAEMOPROTEUS COLUMBAE*

B.1 Abstract

There was a need to develop primers for PCR and quantitative-PCR for *Haemoproteus columbae*, a malaria parasite of Rock Pigeons *(Columba livia)*. Previously published primers for *Plasmodium, Parahaemoproteus* and *Haemoproteus* did not amplify *H. columbae* efficiently. Several primer sets were developed to amplify fragments of the mitochondrial cytochrome oxidase I gene of *H. columbae* for use in both PCR and qPCR. Protocols were also optimized for the *C. livia* gene CHD1W for use in PCR, and primers were also developed for *C. livia* GAPDH for use in qPCR. These primers will be useful in the future for rapid prevalence and abundance surveys of *H. columbae* in feral and domestic pigeon populations.

Keywords: Haemoproteus columbae, Pseudolynchia canariensis, Columba livia, *pigeon, malaria, parasite*

B.2 Introduction

The development of *Haemoproteus columbae*-specific primer sets was necessary as previously published primers for *Plasmodium, Parahaemoproteus* and *Haemoproteus* did not amplify the pigeon malaria parasite *H. columbae* under the published PCR conditions efficiently (Escalante et al. 1998, Perkins and Schall 2002, Martinsen et al. 2006, Martinsen et al. 2008). Many published reactions required a two-step process, first amplifying a larger DNA fragment using an "outer" primer set, then amplifying a smaller DNA fragment within the larger fragment with an "inner" reaction. Here, I describe a single reaction process to amplify *H. columbae*. I also describe modifications to a published PCR protocol using the primers Pf2550 and Pr2718 (Fridolfsson and Ellegren 1999) to amplify Rock Pigeon *(Columba livia)* DNA. These primers are useful to check

the success of DNA extractions and confirm that a given pigeon is negative for *H. columbae*. These primers have the additional benefit of providing different fragment lengths for male and female pigeons as it amplifies a variable region on the W sex chromosome of a gene called CHD1W, and so the sex of birds can be determined from this PCR reaction.

B.3 Methods

I developed all other primers for *H. columbae* and *C. livia* using published sequences available on the NCBI Nucleotide website (http://www.ncbi.nlm.nih.gov/nuccore/) and using the software Primer3 (Rozen and Skaletsky 2000). I also developed a Quantitative RT-PCR (qPCR) reaction protocol to detect the relative abundance of the malaria parasite *H. columbae* in a blood sample of *C. livia*. Blood samples were collected from pigeons by brachial venipuncture and blood dots were preserved on filter paper and kept at -20 °C before processing. DNA extractions from blood dots followed the standard protocol provided in a DNeasy Blood and Tissue Kit (Qiagen).

B.3.1 PCR

Regions of the mtDNA cytochrome oxidase I (CO1) gene of *H. columbae* were amplified using two primer pairs CO1529 with CO1886 (~350 bp) and Co1523 with Co1746 (~225 bp). All primer sets are shown in Table B.1. PCR reactions for *H. columbae* were carried out in a total volume of 25μL using 1μL of each primer diluted to 10μM concentration, 2.5μL 10x Standard Buffer, 0.125μL taq (New England BioLabs), 0.5μL dNTPs, and 17.875μL H_2O, with 2μL DNA template at 50ng/μL concentration. Amplification conditions of CO1 for *H. columbae* included an intial activation step at 94

°C for 4 minutes, followed by 35 cycles of 94 °C for 20 seconds, 57 °C for 30 seconds, 68°C for 50 seconds, and a final extension of 68 °C for 7 minutes.

The CHD1W gene of pigeons was amplified with published primers 2550F and 2718R (Fridolfsson and Ellegren 1999) in a total reaction volume of 10μL using 1μL of each primer diluted to 10μM concentration, 1μL 10x Standard Buffer, 0.05μL taq (New England BioLabs), 0.25μL dNTPs, and 5.7μL H$_2$O, with 1μL DNA template at 50ng/μL concentration. Amplification conditions of CHD1W for *C. livia* included an intial activation step at 94 °C for 2 minutes, followed by 10 cycles of 94 °C for 30 seconds, 60 °C 30 seconds (decreasing 1 °C each cycle), 72 °C 40 seconds. These cycles were then followed by 34 cycles of 94 °C for 30 seconds, 50 °C 30 seconds, 72 °C 40 seconds, and a final extension of 72 °C for 5 minutes. The gene fragment for male pigeons (~3000 bp) is larger than the fragment for female pigeons (~1700 bp) by approximately 1300 bp.

B.3.2 Quantitative RT-PCR (qPCR)

To estimate the relative number of copies of parasite genomes to host genomes, and thus estimate infection intensity for *H. columbae*, a quantitative PCR (qPCR) assay was developed. DNA was extracted as for PCR above. The presence of high quality DNA was confirmed by spectrophotometric analysis (Nanodrop Technologies). GAPDH is a reference gene that is routinely used to normalize between qPCR samples to control for concentration difference between samples in a given experiment (Munson et al. 2008, Kubinak et al. 2012). In this case, the normalized relative ratio of pigeon GAPDH was compared to that of *H. columbae* to determine the relative abundance of *H. columbae* in the blood, which can be interpreted as infection intensity. Primers for a region of exon 8

of the pigeon GAPDH gene and new primers for a shorter fragment of the CO1 gene of *H. columbae* were again developed from published sequences as before (Table B.1).

The qPCR reaction was run using a Roche Lightcycler ® 2.0 system with the capacity for 32-LightCycler® Capillary reactions arranged in a carousel. Reagents were purchased from Roche (product no. 05140340001) that included the reporter SYBR Green as part of the LightCycler® FastStart DNA Master^{PLUS} SYBR Green I kit (Roche). Reactions were optimized for a 10µL final reaction volume. Settings for optimal reaction conditions are provided in Table B.2. Reactions for both GAPDH and the smaller *H. columbae* fragment of CO1 (using primers CO1F529 and CO1R746) were each run with 6µL HPLC H$_2$O, 0.5µL of each primer diluted to 10µM, 2µL Taq, and 1µL DNA template. Reactions were paired with each *H. columbae* reaction paired with the GAPDH *C. livia* reaction for the same sample so that the normalized relative ratio of parasite to host DNA could be calculated following the reaction. All reactions were followed by a melt analysis, which measured fluorescence intensity every 0.1 °C increase from 65 °C to 95 °C, and a final cooling step down to 40 °C. Initially qPCR reactions were run in triplicate, but there was very minimal variation among replicated samples, and so later samples were run singly and any problematic samples were run again.

To accurately interpret qPCR results and control for any differences in PCR efficiencies between the primer sets, calibration curves were generated for each reaction using varying concentrations of DNA template (1:1 to 1:256 in serial dilutions) for an infected pigeon. A standard calibration curve allowed for comparison across various DNA concentrations in later runs. Methods and interpretation of the qPCR assay were

Table B.1. Primer pairs developed for *Haemoproteus columbae* and *Columba livia* for PCR and qPCR.

Organism	Primer sequence (5'-3')	Amplified gene	Primer name	Works best with Primer(s)	Use for
H. columbae	CATTTACGAGC AAAAGGACTAA CAT	CO1	CO1F529	CO1R886	PCR
H. columbae	GGGCCCATACA ATACTTCCT	CO1	CO1R886	CO1F529	PCR
C. livia	CCATCACAGCC ACACAGAAG	GAPDH	ropiGAPF14	ropiGAPR152	qPCR
C. livia	CCATTGAGCTC AGGGATGAC	GAPDH	ropiGAPR152	ropiGAPF14	qPCR
H. columbae	GTTGTACATTT ACGAGCAAAAG G	CO1	CO1F523	CO1R746	qPCR
H. columbae	TCAGGATGACC AAAGAACCAG	CO1	CO1R746	CO1F523 or CO1F529	PCR or qPCR

Table B.2. qPCR cycle settings for CO1 of *H. columbae* and GAPDH exon 8 of *C. livia*

Default Channel = 530
Seek temp = 30
Max seek pos. = # of samples
Instrument type = 3 Channel (530=SYBR Green)

Target C	Hold	Ramp Rate	Aq. Mode	Program Name	Cycles	Analysis Mode
95	10min	20	None	Denature	1	None
95	10sec	20	None	PCR 3 step	55	Quantification
57	3sec	20	None			
72	9sec	20	Single			
95	0sec	20	None	Melt	1	Melting Curves
57	15sec	20	None			
95	0sec	0.1	Continuous			
40	0sec	20	None	Cool	1	None

modeled on those of Kubinak et al. (2012). Negative (no template) and positive controls were run in each reaction, with the same calibrator sample serving as the positive control for both *H. columbae* and *C. livia* reactions so each run could be calibrated. Next, normalized relative ratios of the DNA of host and parasite were compared using the Roche LightCycler® 2.0 software, first selecting calibration curves to control for reaction conditions. Next, paired host (GAPDH) and parasite (CO1) samples were compared to normalize values for concentration differences to calculate the normalized relative ratio. Although the gene fragment for *H. columbae* was ~225 bp, and the GAPDH *C. livia* fragment was ~100 bp, greater AT nucleotide bias in the *H. columbae* fragment (72% at in *H. columbae*, 44% in *C. livia*) caused the melting temperature to be lower for the larger fragment. *C. livia* GAPDH peaked at nearly 86°C (85.75-85.97), while *H. columbae* CO1 melting temperature peaked close to 75°C (75.47-75.73). The no-template control melting temperature was easily distinguishable at 67-68°C.

The infection status of *C. livia* samples with *H. columbae* was determined by comparing the crossing point (Cp), which is the first cycle where the amount of DNA is detectable and read by the laser in the LightCycler. A lower Cp means more DNA is present since this point is reached faster. A higher Cp means that less DNA is present and the reaction takes longer, or more cycles to reach the Cp. This value is dependent on the reaction conditions and the particulars of the DNA, but generally Cp less than 25 to 30 is a positive result, and Cps that are greater than 30 are likely negative or contaminated, or very low level DNA. The results of *C. livia* were typically between 25-30 and thus easily interpretable, but the Cp of *H. columbae*, as might be expected given that there is more variation in parasite infection intensity across hosts, were more variable. It was often

useful to examine the melting temperatures of questionable reactions and if they were not within a degree or two of the normal melting temperature range for this CO1 *H. columbae* gene fragment, then the sample was considered negative.

B.4 Discussion

The extremely sensitive detection capability of the qPCR reaction is both a benefit and a reason for caution in using this technique. The most accurate and repeatable (not to mention least expensive) way to compare infection intensities of *H. columbae* in *C. livia* may still be by scanning blood smears and counting the number of parasites, the main drawback being a larger investment of time compared to molecular methods.

B.5 Acknowledgements

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B.6 References

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APPENDIX C

H. COLUMBAE SEX RATIOS AND THE RELATIONSHIP

BETWEEN DOUBLE GAMETOCYTE INFECTION

AND INFECTION INTENSITY

C.1 Introduction

The distribution of blood parasites in host cells may influence their transmission dynamics. In the case of malaria parasites, the sexual stage of reproduction occurs within a blood-feeding insect, such as a mosquito or hippoboscid fly. The parasite cannot be transmitted to a new host if it does not mate in the insect. If the density of malaria parasites is low enough in the blood of the host, there is a chance that male and female parasites might not find one another once taken up in a blood meal by the insect, and may not successfully mate. One hypothesis is that parasites are nonrandomly distributed in the blood cells of the infected vertebrate host, such that males and females occupy the same host red blood cell more frequently than would be expected by chance (Jovani and Sol 2005). If this were so, then when males and female gamete precursors (gametocytes) shared a blood cell, it would facilitate males finding female parasites in an insect blood meal. Since parasite fertilization is a major bottleneck for transmission, parasites with this strategy might have higher fitness than parasites without this strategy (Jovani 2002). Other infection dynamics, such as the ratio of male and female parasites in a given infection, may also influence whether this is a successful strategy. One prediction is that with increasing gametocyte density in an infection, the sex ratio should become more male biased, assuming that males were not genetically identical and were in competition for females (larger sex ratio) (Neal and Schall 2010). Here, I investigate the infection dynamics of *Haemoproteus columbae*, a malaria parasite of feral Rock Pigeons *(Columba livia)*, to determine whether parasites more frequently invade the same host cell than would be expected by chance. I also test whether parasites are more likely to share a cell

is based on the proportion of male parasites (sex ratio) of an infection, or the maturity of the infection (ratio of young to older parasites).

C.2 Methods

Blood smears from 26 Rock Pigeons *(Columba livia)* infected with the malaria parasite *Haemoproteus columbae* were examined. Blood smears used in this project were pre-screened and only smears with an average of at least one parasite per microscopy field at 1000x were used in this study. Smears were observed at 1000x using an oil immersion lens to determine parasite infection intensity. Blood cells in each microscopy field of view were counted for a minimum of 300 blood cells (1-3 microscopy fields depending on cell density), and the number of parasites occupying these cells was quantified to estimate infection intensity. Only areas of a prepared smear where the cells were nonoverlapping were used, and cells were counted only if the whole nucleus of the cell was visible. When more than one parasite occupied a cell, this was still counted as one infected blood cell.

After infection intensity data were collected, a new section of the smear was examined and demographics were recorded for the first 50 parasites encountered. Age was estimated by size, where parasites needed to be larger than the host cell nucleus to be considered mature (smaller were immature). Each time two parasites occupied one cell (a double gametocyte infection, or DGI), or in one rare case three parasites in a single cell (triple gametocyte infection, or TGI) the demographics of the parasites sharing the cell were categorically recorded as combinations of male, female, or immature (sex cannot be determined) parasites.

C.3 Results

About half of the blood smears examined revealed double gametocyte-infected cells (12 / 26 smears). A mean of 333.8 cells was examined per smear (range 300 - 391) for a total of 8,680 cells across all smears. A total of 143 infected cells was found (range 1 – 41 per smear). The average percentage of infected cells was 1.63% (range 0.28% - 12.54%). Only one TGI was found and so it was excluded from data analyses as an anomaly. Information about the parasites that were in doubly infected cells is shown in Table C.1. The most common type of DGI found was two male gametocytes sharing a cell (33%), followed by two immature gametocytes (24%). Male and female or male and immature combinations were 15% of DGIs.

The likelihood of finding DGIs increased with parasite density in the infection (Figure C.1; regression r-square = 0.28, P = 0.006). Sex ratio was highly variable across infections; male parasites ranged in proportion from 0.05 to 0.76 (Figure C.2).

C.4 Discussion

Similar work has been done on *H. columbae* but only for 4 pigeons (12 smears total) (Jovani and Sol 2005), and the authors found that DGIs were more frequent for *H. columbae* than would be expected by chance. In the current study, just under half of the 26 infections we examined had DGIs, and DGIs were more likely to be found with higher density infections. Jovani and Sol (2005) found DGIs in every infection examined regardless of infection intensity. However, these authors examined at least 2600 blood cells in each pigeon smear and examined a minimum of 500 infected cells. In contrast, a mean of 333 cells was examined for each infection in this study (total 8,680 cells) and

Table C.1. Information on DGI infections. M = male gametocyte, F = female gametocyte, I = immature gametocyte (sex could not be determined). Pigeon ID is a unique identifier for a particular infected pigeon blood sample.

Pigeon ID	M,F	M,M	F,F	M,I	F,I	I,I	Total DGIs
2059		1				4	5
2064							0
2065							0
2066	1	1				1	3
2068	1	1		2	1		5
2069						2	2
2219							0
2226	1	1					2
2230							0
2238		1					1
2248							0
2256	1	2					3
2259			1				1
2260		2		3			5
2263							0
2265							0
2266							0
2271							0
2283							0
2284							0
2285							0
2286							0
2288							0
2292	1	1					2
2295			1			1	2
2300		2					2

Figure C.1. The proportion of infected cells that were infected with more than one parasite increased with intensity of infection. Parasite intensity was nonlinear and is shown on a log scale.

Figure C.2. Sex ratios of *H. columbae* in its natural host *C. livia*. Data are from 26 infections. A reference line (dotted) is included at the mean value (0.486).
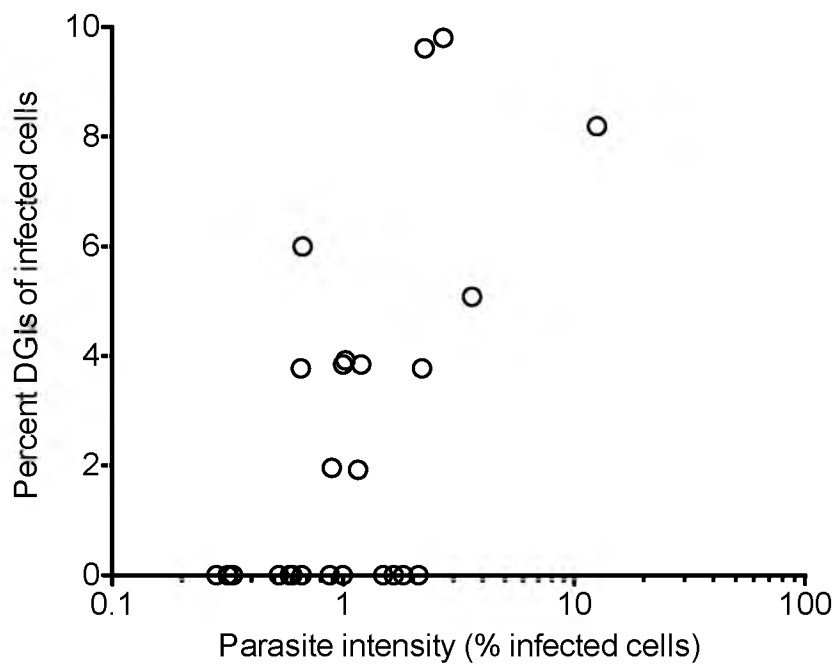
resultantly fewer infected cells were found (total 143), and it is likely that DGI prevalence was underestimated since relatively few cells were examined and multiply infected cells are uncommon.

TGIs are uncommon across all malaria parasite species (Jovani et al. 2004) and the results of this survey also found that TGIs were rare for *H. columbae*, finding only 1 in 26 infections. The occurrence of mature male-female DGIs was only 15%. This proportion seems quite low, yet compared to a literature survey of other *Haemoproteus* species where DGIs were found, only 4.2% of DGIs were male-female combinations (Jovani et al. 2004). This suggests that *H. columbae* may have higher male-female DGIs than most other malarias where they have been looked for. 15% is a somewhat conservative estimate given that some of the female-immature, male-immature, and immature-immature combinations could have later developed into male-female DGIs. Unfortunately in a previous study of *H. columbae* DGIs the sex of the co-habitating parasites was not provided (Jovani and Sol 2005), and so comparisons cannot be made directly to determine if DGIs for *H. columbae* would typically provide a benefit of increased mating success for the parasite once taken up by an insect.

It is interesting to note that the most common combination was male-male gametocytes in DGIs (33%). The occurrence of two males in one parasitized cell would potentially only provide further competition for mates and resources for the males sharing the cell with no obvious fitness benefit. This combination could be explained as error from a small sample size, but it would be worth pursuing to find out if this is a real phenomenon. Perhaps male parasites mature first, or are more likely to invade the same cell than females. Cohabitation by females was very low in comparison with this

combination representing only 6% of DGIs. The sex ratio of the male and female parasites in the infections does not explain this difference. The sex ratio was a mean of 0.486, very close to 0.5, and typical of that of sex ratios for other malaria parasites (Neal and Schall 2010).

While further investigation of more DGIs is necessary to determine if the male-female combination of gametocytes is as rare as it seems, it would be very interesting to pursue. If the combination of heterogeneous gametocyte sexes is rare, it suggests that there is not actually any benefit to DGIs as has been previously suggested (Jovani 2002, Jovani et al. 2004). Alternatively, DGIs may be detrimental, causing further competition for either mates (for males) or resources in the blood cell, and might be explained by coincidental co-infection of the same cell by the parasite, perhaps if the rate of parasite is loosely synchronized by the eventual sex of the gametocyte. If DGIs do occur in host cells more often than would be expected by chance (Jovani and Sol 2005), it may be a consequence of the synchronized life cycle of the parasite. In the case of *H. columbae* a small portion of a pigeon's blood cells are more likely to be infected if these particular blood cells happen to be in the epithelial lung tissue as parasites invade the peripheral blood.

C.5 Acknowledgements

heavy infections by either J.L.W., Autumn R. Henry, or Adrian(ne) Smith in addition to

SKH.

C.6 References

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APPENDIX D

ROCK PIGEON HEMATOCRIT IS AFFECTED BY AGE

AND TIME IN CAPTIVITY, BUT NOT BY

H. COLUMBAE

D.1 Introduction

Hematocrit is the density of red blood cells in the fluid blood, often measured as the proportion of packed blood cells of the total blood volume. Hematocrit has been used to assess body condition, where healthier individuals typically have a higher hematocrit. The link between body condition and hematocrit is not always clear, although there have been some studies that support this connection. For example, in food stressed individuals, hematocrit typically drops because energetic reserves are insufficient for making new red blood cells (Bearhop et al. 1999). While there is some evidence that lowering hematocrit by the removal of blood cells can decrease body condition (Brown and Brown 2009), whether the opposite is true, that healthier individuals have higher hematocrit has seldom been tested in nature.

In birds, it is assumed that there is a positive relationship between body condition and hematocrit. However, the validity of this assumption may be difficult to judge because the baseline hematocrit levels of many avian species are unknown (Dawson and Bortolotti 1997); therefore the comparative analysis of hematocrit is limited. Studies of Gray Catbirds *(Dumetella carolinensis)* suggest that while individual hematocrits are repeatable across years for birds caught in the same season, hematocrit varies both by season and by the number of times a bird is captured (Hatch and Smith 2010). Hematocrit was not related to body condition in nestling Tree Swallows *(Tachycineta bicolor)* but was instead largely influenced by the environment (Morrison et al. 2009).

Another factor that may influence the hematocrit of birds is blood parasites, particularly the avian malaria parasites. The negative effects of malaria infection vary with parasite species (Atkinson and van Riper 1991, Valkiūnas 2005), but some can

cause anemia (Hill 1942) and some species of avian malaria parasites decrease body condition (Yorinks and Atkinson 2000, Atkinson et al. 2008). The assumption that malaria parasites, including those in the genus *Haemoproteus*, decrease avian hematocrit has not often been tested. In a study of American Kestrels *(Falco sparverius)*, birds with *Haemoproteus* had higher hematocrit levels than uninfected birds (Dawson and Bortolotti 1997), which is opposite the prediction. This is surprising because parasites often destroy blood cells and the host immune system may also actively remove infected blood cells; both would lower hematocrit. However, if the host either rapidly replaces destroyed cells or becomes dehydrated by the infection, hematocrit may actually increase.

The inconsistent results of previous studies show that hematocrit is variable and may be an unpredictable indicator of body condition. In this study we examined factors that might influence the hematocrit of feral Rock Pigeons *(Columba livia)* to better understand what causes hematocrit to differ between individuals. We investigated whether time spent in captivity, sex, age, or infection status and intensity (density of parasites in the blood) of *H. columbae* influenced hematocrit in pigeons.

D.2 Methods

D.2.1 Blood collection

Blood samples were taken from 86 Rock Pigeons that had been (1) recently trapped (bled < 4 days since capture, N = 36), (2) kept in long-term captivity (several months to several years, N = 39), or (3) kept in long-term captivity and were host to populations of hippoboscid flies (N = 11). The sex (if known), approximate age, and time spent in captivity were recorded for each pigeon at the time of blood sampling. Pigeons were determined to be juvenile (< 6 months old) if they had a grey cere and brown eye color;

very young pigeons were "squeakers" with distinct vocalizations that indicated they had recently fledged. Pigeons older than 6 months are adults (Johnston and Janiga 1995). Blood was taken by brachial venipuncture and collected into three heparinized 70 μL capillary tubes. We used the blood to measure hematocrit and make duplicate blood smears to later examine them for malaria parasites. Triplicate capillary tube samples were taken to ensure samples were not lost during later steps in preparing or measuring hematocrit, and when multiple samples were collected (at the same time) they were averaged to improve accuracy.

D.2.2 Preparation and examination of thin blood smears

Small drops of blood were put onto duplicate frosted-end glass microscope slides and thin blood smears were made. The capillary tubes were then sealed at one end with Critoseal clay putty and placed on ice. Blood smears were dipped in methanol for one minute, and stained with a dilute solution of Baker brand Giemsa stain and buffered distilled water (1:10, pH 7.0) for 50 minutes, then rinsed in buffer and air dried before examination. Smears were examined under microscopy at 1000x using an oil immersion lens. To estimate the density of *H. columbae* parasites in the blood of a given bird, parasites were counted in 25 different microscopy fields of view at 1000x magnification, using only areas of the slide where cells were evenly distributed and blood cells didn't overlap one another.

D.2.3 Hematocrit

Capillary tubes were sealed at the end opposite the clay plug by melting the glass using a Bunsen burner. Care was taken to melt the glass without forming a bubble (which the glass will do if overheated) and without heating the blood sample itself. The capillary

tubes were labeled with a small flag of tape and then loaded around the edges of 10mL centrifuge tubes. The center of the 10 mL centrifuge tubes was packed with foam cores to keep the capillary tubes in place. The centrifuge tubes were then spun at 3,000 rpm for 10 minutes to separate the blood cells from the serum layer. Once the blood was separated, a specialized hematocrit scale was used. The scale is held under the blood-filled microhematocrit tube at the appropriate level for the total volume of liquid, and the ratio of packed red blood cells to serum was compared to give a measure of hematocrit. When samples were intact in duplicate or triplicate the average value was used. When multiple hematocrit samples were available, more than one individual researcher trained in this protocol took readings and the values were averaged.

D.3 Results

There was no overall difference in hematocrit between juvenile (N = 11) and adult (N = 75) pigeons (Mann Whitney test, U = 351.0, P = 0.40). However, the amount of time spent in captivity strongly influenced hematocrit, with recently caught birds having a higher hematocrit (Figure D.1, Mann Whitney test for all birds, U = 609.5, P = 0.008; for adult birds only U = 330.0, P = 0.007).

Thus, comparisons between pigeon age and hematocrit were re-analyzed for only the pigeons recently caught and maintained in captivity (pigeons in the longer-term captive groups were no longer juvenile and no equivalent comparison could be made). We found that recently caught older and younger pigeons did differ significantly in hematocrit, with older pigeons having a higher hematocrit than younger birds (Figure D.2, Mann Whitney test, U = 69.5, P = 0.02). We tested whether this difference in hematocrit was due to differences in *H. columbae* prevalence or intensity by comparing these measures between
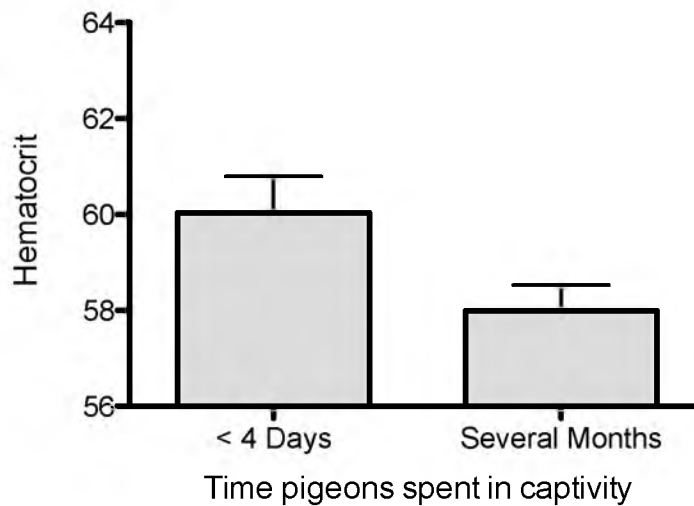
Figure D.1. Pigeons that had spent less time in captivity had a higher average hematocrit value (proportion of the blood volume that is comprised of blood cells). Values shown are for mean hematocrit + SE.

recently caught juvenile and adult pigeons. Adults were more likely to be infected than juvenile pigeons (Figure D.3; Chi-square, $P = 0.03$).

However, only two of 11 juvenile pigeons were infected, and so I could not determine whether there was an effect of the intensity of infection, or whether there was an effect of infection on hematocrit across pigeon age classes. Of the recently caught adult pigeons, I compared the hematocrit of those that were infected with *H. columbae* to those that were not. I found no difference in hematocrit due to parasite prevalence (Mann Whitney test, U = 54.0, $P = 0.32$), and of the infected birds, parasite intensity was not correlated with hematocrit (r-square < 0.001, $P = 0.96$). I repeated these analyses for long-term captive adult pigeons and found no difference in hematocrit due to parasite prevalence (Mann Whitney test, U = 247.0, $P = 0.99$), and of the infected birds, parasite intensity was not correlated with hematocrit (r-square = 0.02, $P = 0.65$).
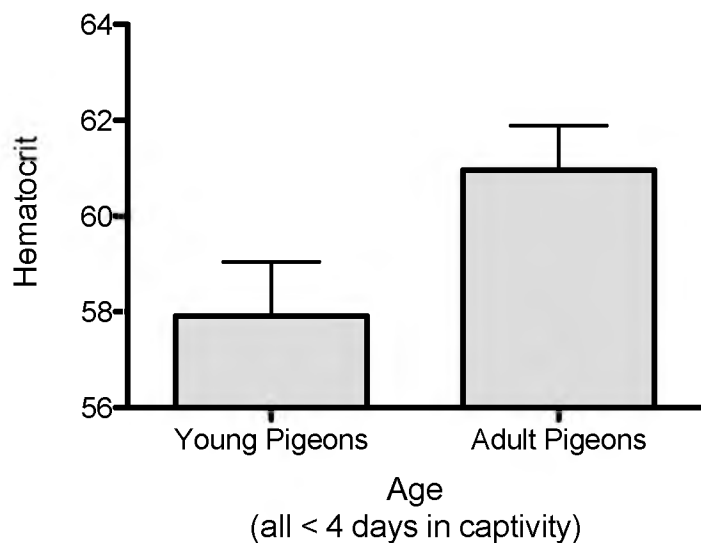
Figure D.2. Juvenile Rock Pigeons had a lower average hematocrit than adults. Values shown are for mean hematocrit + SE.
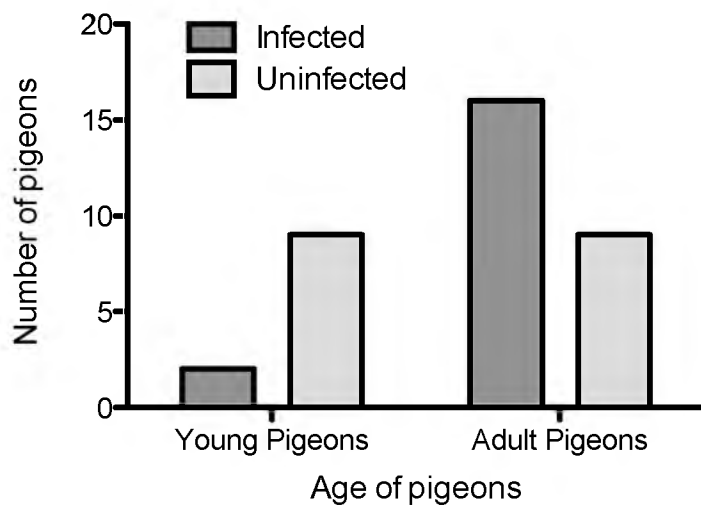

Figure D.3. Adult (> 6 months of age) Rock Pigeons were more likely to be infected with the malaria parasite *H. columbae* than younger birds.

As expected, since pigeons could not be re-infected with *H. columbae* while in captivity, recently caught adult pigeons were more likely to be infected with *H. columbae* (Chi-square, P = 0.002) than adult birds maintained for at least one month in captivity. However, of the birds that were infected, time in captivity did not significantly influence the intensity of *H. columbae* infections (Mann Whitney test, U = 67.5, P = 0.11). Not enough pigeons were sexed to determine if sex influenced hematocrit. There was no effect of hippoboscid flies feeding on the hematocrit of captive birds for several months (long-term adult pigeons only were compared; Mann Whitney test, U = 302.0, P = 0.95).

D.4 Discussion

Pigeons kept in captivity lose their *H. columbae* infections over time. Thus, it is not surprising that recently caught pigeons were more likely to be infected with *H. columbae* than the long-term captive birds in this study. It was unexpected that recently caught adult pigeons were more likely to be infected with *H. columbae* than younger pigeons given that previous work suggests otherwise (Sol et al. 2003). The different results found in our study may be explained by either smaller sample sizes or differences in sampling season. All "recently caught" pigeons in this study were sampled in early June; infections in younger birds are often more common later in the summer and into fall when the fly populations that vector the parasite increase (Klei and DeGuisti 1975). We did were not able detect seasonal patterns of infection for pigeons in the Salt Lake City, UT area, possibly due to low sampling, particularly during the late summer months. It is possible that there is less seasonal variation in this region for other reasons that we have not yet determined (Appendix A).

Interestingly, *H. columbae* infection has no influence on pigeon hematocrit or vice versa. This result is surprising given the assumption that malaria parasites destroy red blood cells, and thus that infection should lower hematocrit. We found similar results when infection intensity, pigeon age and/or time in captivity were considered as well. Our results contradict other studies that have found an effect of malaria in the genus *Haemoproteus* on avian hematocrit. American Kestrels had increased hematocrit when infected with *Haemproteus* parasites (Dawson and Bortolotti 1997); alternatively, a study on Blue Jays found that *Haemoproteus danilewskyi* only decreased hematocrit for one week of an 8-week study (at week 5 post infection), and there was no overall effect of the parasite on packed cell volume (i.e. hematocrit) (Garvin et al. 2003). It could be that the impact of *Haemoproteus* infections on hematocrit is system specific.

H. columbae in Rock Pigeons was observed to cause anemia and "watery blood" in heavily infested pigeons, suggestive of low hematocrit (Markus and Oosthuizen 1972). Similar blood pathology, as well as extensive tissue pathology, was also observed in the Bleeding Heart Dove *(Gallicolumba luzonica)* infected with *H. columbae* (Earle et al. 1993). It seems possible that pathology of *H. columbae* is density dependent, where only exceptionally heavily parasitized pigeons show symptoms including reduced hematocrit, while less heavily infected birds are able to generate new blood cells at a rate that keeps pace with those lost from infection. Considering pigeons replace their blood cells every 35 days (Voss et al. 2010), this may explain our results. Additionally, it is also possible that the loss of blood cells due to malaria infection is not a large percentage of the bird's total blood volume. The Animal Care and Use Guidelines for taking bird blood samples recommends taking no more than (but up to) 1% of a bird's mass in blood at a time,

apparently a volume that has no dire health consequences for the bird (Gaunt and Oring 1999). It remains unclear whether *H. columbae* destroys more than that 1% of the volume of the bird's blood cells, as no effect on hematocrit was detectable.

Another possibility is that hematocrit is not a good estimate of avian condition and physiology, as others have suggested (Dawson and Bortolotti 1997, Bearhop et al. 1999, Potti 2007). The pathology associated with *H. columbae* infection might not be detectable with a test of hematocrit, and rather tissue pathology could be much more important (Earle et al. 1993), as has been shown for pathology of *H. meleagridis* in turkeys (Atkinson and Forrester 1988).

Hematocrit is thought to vary with hydration (Fair et al. 2007), which could partially explain why recently caught birds had higher hematocrit than those that had remained in captivity longer. Often it takes pigeons a couple of days to adjust to captivity and feed and drink normally *(pers. observation)*, possibly explaining this difference. It is also possible that recently caught pigeons had experienced greater activity levels that may have impacted hematocrit, though because physical activity can cause greater wear on red blood cells, greater activity levels would more likely lower hematocrit. While we were not able to determine the sex of many of the pigeons to compare hematocrit, sex is not an important indicator of hematocrit in avian species (Fair et al. 2007).

The difference in hematocrit between younger and older pigeons, with older birds having a higher hematocrit, is what we expected based on other work examining avian physiology. Our results were similar to previous studies using pigeons and other avian hosts, suggesting that our measurements of hematocrit were accurate (Fair et al. 2007, Prinzinger and Misovic 2010).

In conclusion, the results of this study show that there is no effect of *H. columbae* infection on the hematocrit of pigeons, suggesting that hematocrit is not an accurate indicator of bird condition. Even though hematocrit does seem to be robustly associated with host age, it is likely influenced by other factors such as hydration level and stress. Taking avian hematocrit and garnering useful information about bird condition seems problematic.

D.5 Acknowledgements

This work was conducted as an independent research study project at the University of Utah by Ben Ku with the assistance of Autumn Henry and my help and supervision. Thanks to Jason Kubinak for showing us how to measure hematocrit using the lab equipment that was available.

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APPENDIX E

CONSTRUCTION AND USE OF PIGEON BACKPACKS

E.1 Introduction

I describe the use of backpacks for priming the pigeon immune system against flies in Chapter 3; however, here I provide a detailed account of their construction for use by future researchers. The backpacks were used to contain small or even single fly populations on a particular pigeon host, and this technique could be used in many experiments ranging from giving pigeons a controlled exposure to infected or uninfected fly bites, to monitoring fly life history, to generating and tracking particular fly genotypes or phenotypes for several populations without the time-intensive hassle of having to ruffle or expose the entire body of the host pigeon to CO_2 to recover all flies.

This is not the first time that flies have been exposed to pigeons using backpacks. Coatney (1931) described a brass breeding cages 1 ½ inches in diameter and ¾ inches high with screw tops and bottoms fitted with wire screens; these were affixed to pigeons' backs with small wires around the wings. Mohammed (1958) used cardboard pillboxes with elastic straps to fit around the pigeon wings, and a sort of muslin cheesecloth gauze netting on one side. While these containers are sufficient, neither small brass containers, nor cardboard pillboxes, are easy to come by these days. I provide detailed instructions for making durable and reusable backpacks with easy to find materials.

E.2 Methods

The backpacks were constructed using urine sample containers acquired from local health clinics. The lids were removed from the containers and they were cut to approximately 1.5" high from the base of the container using a hacksaw. A dremel tool fitted with a drill-like small metal bit was used to create a 1"cm diameter hole in the base of the cups where later flies could be added one at a time and the hole covered by a

fingertip to prevent them from escaping. Two pairs of small holes were added using a dremel tool close together and low on the opposite open sides of the container. These holes were made just large enough to fit small beading elastic. Lengths of beading elastic at least 6 inches long were threaded through the pairs of holes to later provide two places where the elastics could be tied around the wings of a pigeon to keep the backpack in place. Small circles of fly-proof netting (small mesh size, approx. 2mm) were cut from the netting fabric to fit the open side of the container, these were held in place with rubber bands. The netting allowed flies to make the best contact for feeding on the back of the pigeon when it was not completely flat, and so an uneven surface was created by bunching the netting to make the surface that would contact the bird variable to increase the chance that flies could make contact. The netting was glued in place using a craft glue gun, carefully sealing all around the edges of the fabric, and around the holes for the elastic to keep flies from escaping. An example of an empty backpack is shown in Figure E.1, and a backpack on a pigeon is shown in Figure E.2.

E.3 Results and Discussion

To use the backpacks, flies were placed inside of the pack via the small opening on what was originally the base of the container (now the top of the container if the backpack is oriented for use of feeding flies on a pigeon). Clear plastic tape (such as Scotch brand) was used to keep flies in the container, first taping a small piece of the tape to itself over the opening such that the flies could not stick to the tape at this point, but so that the tape sealed completely surrounding the opening. Backpacks were then ready for
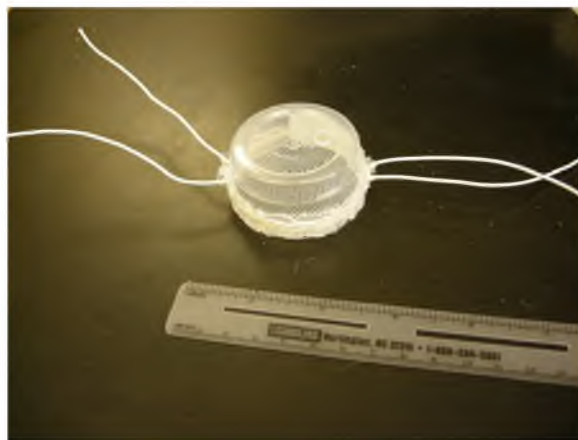
Figure E.1. A "backpack" can be used to contain hippoboscid flies on a pigeon. Mesh netting on the bottom of the backpack allows the flies to feed on the back of a bird when the feathers have been removed from the area of contact. Flies can be observed through the container and replaced using a hole in the top of the container as needed. The hole in the top of the container is covered with a small piece of scotch tape to prevent flies from escaping. The segment of tape that faced the inside of the container was covered with another small pieces of tape to prevent flies from sticking to the tape.

Figure E.2. A Rock Pigeon wearing a backpack. Note that ends of the elastic tie straps have been taped over with duct tape to prevent the pigeon from untying the knots that hold the backpack in place.

fitting onto pigeons, taking care to pluck away only the region of the back feathers that would be in the way of the backpack contact zone. Feathers re-grew after 1-2 weeks on average and needed to be removed as soon as they formed pins as even these small feathers would push the backpack far enough away from the bird's skin as to prevent flies from feeding. Elastic was tied around the wings of pigeons, taking care not to make it too tight, and tying the knots behind the wings. Knots were visible on the outside of the bird's feathers to prevent irritation of the knot under the wing and to make them less accessible to preening pigeons. I found pigeons to be expert in untying knots; no matter how many knots I made, the pigeons had no trouble getting them loose. Thus, all knots were taped over with duct tape to prevent birds from getting access to the ends of the elastic and unraveling the knots.

Backpacks were used for up to two weeks for the purposes of the experiments in this dissertation, but could be used for longer as long as they were monitored daily. Issues with backpacks included birds flipping them over (which if not caught early often killed the flies in the backpack if they could not feed for more than 24 hours), pulling holes in the netting (rare), and flies not being able to reach the bird skin due to either feather regrowth or issues with the landscape of the netting preventing them from being able to access the bird skin. Flies could be replaced easily in the pack as needed, and monitored often by tilting the pack to view them through the netting even while the backpack remained on the bird. I think this technique could be very useful if monitoring individual flies or particular fly-host combinations is necessary as it prevents the labor-intensive process of lengthy ruffling or CO_2ing of the whole bird to find the flies.

E.4 Acknowledgements

E.5 References

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