IMPACT OF AN INTRODUCED PARASITE ON DARWIN'S FINCHES

by

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STATEMENT OF DISSERTATION APPROVAL

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ABSTRACT

Invasive parasites are a growing problem as humans continue to traverse the globe. The impact invasive parasites have on naïve host populations is the focus of my dissertation. To date, the Galapagos Islands remain one of the most well-preserved archipelagos, with no known extinctions of endemic bird species. However, the recent introduction of *Philornis downsi*, an obligate nest parasite, threatens birds across the islands, including the iconic Darwin's finches.

Using an experimental manipulation of parasite abundance in nests, my work shows the detrimental effect *P. downsi* has on fledging success in medium ground finches (*Geospiza fortis*). I explore the mechanisms underlying these effects by investigating the impact of *P. downsi* on nestling growth and condition. I demonstrate that adult medium ground finches and seven other species of Darwin's finches produce *P. downsi*-specific antibodies. Nestling medium ground finches did not have detectable *P. downsi*-specific antibodies nor was there evidence of maternally transferred antibodies. Parental behavior also changed in response to *P. downsi* parasitism, though neither immunological nor behavioral responses were effective against *P. downsi*, and did not result in increased host reproductive success. Finally, using data from my three-year study, I present a model that predicts population viability of medium ground finches in light of the observed effects of *P. downsi* on host fitness. The model predicts that medium ground

finches on the island of Santa Cruz are likely to go extinct within the next half century unless conservation efforts are able to significantly reduce *P. downsi* populations.

My work highlights the dramatic impact an introduced parasite can have on naïve host populations. Parasites with low host-specificity and high rates of dispersal, such as *P. downsi*, can maintain high levels of virulence. In combination with ineffective host defense mechanisms, introduced parasites can lead to severe host population declines, even extinctions.

TABLE OF CONTENTS

LIST OF FIGURES

Figure

LIST OF TABLES

Table

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My graduate school experience parallels a typical field season in the Galapagos. The extreme heat, cactus spines, fire ants and razor sharp lava rocks present challenges to the tasks at hand. However, upon completion, sitting back with caipirinha in hand, I realize how lucky I have been to experience it all, how grateful I am to those who have helped me, and how much fun I had along the way. Dale Clayton, my advisor and co-author, has always been there with bold ideas, loud gestures, and high expectations. His knowledge and enthusiasm have made me a better biologist and a stronger person. I am very grateful for his continued financial support, encouragement and fearlessness. I am also indebted to the post-docs and graduate students who have made up the Clayton lab over the past five years. I am especially thankful to Sarah Huber, who took me under her wing and showed me the challenges and rewards of doing field work in the Galapagos. It is largely due to Sarah that I am one of the lucky few who will fledge (pending committee approval, of course). I am thankful to Celine LeBohec, Sarah Knutie and Sarah Bush, who were able to join me in the field. I thank them for for their contributions to the projects as coauthors, and also for their perpetual optimism, even while picking spines out of some questionable body regions. I was also very lucky to be supported by wonderful assistants both in the Galapagos (Randy Cordova, Oliver Tisalema, Priscilla Espina, Roger and Mimi Clayton) and in Utah (especially Autumn Henry and Ale Alguilar). I thank my committee, Denise Dearing, Franz Goller, Jeb

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CHAPTER 1

INTRODUCTION

Background

By definition, parasites are costly to their hosts. To minimize these costs, hosts have evolved defense mechanisms that include immunological, behavioral, physiological and morphological adaptations (Clayton et al., 2010, Hart, 1990). In turn, parasites evolve reciprocal adaptations of their own to escape host defenses (Bush & Clayton, 2006, Bush et al., 2010). Hosts and parasites coevolve through this type of arms race such that populations of both groups persist. However, when parasites encounter novel hosts, those hosts may not yet have effective defense mechanisms. In such circumstances, parasites have the upper hand and can have severe effects on host fitness (de Castro & Bolker, 2004).

Observing the dynamics of novel host-parasite associations in wild populations is inherently difficult. For logistical and ethical reasons, experimental introductions of parasites to naïve host populations are usually restricted to laboratory settings. As human populations continue to grow and expand, however, introductions of parasites to novel host populations are becoming more frequent (Smith et al., 2006). Researchers can use these "natural experiments" to study the initial interactions of novel host-parasite associations in wild populations (Lafferty et al., 2005). The accidental nature of most introductions means that the effects of introduced parasites are often not noticed until

host populations begin to decline severely (McCallum & Dobson, 1995), at which point conservation priorities may preclude rigorous experimental study. Thus, it is extremely important that researchers take every opportunity to investigate novel host-parasite associations to better predict the impact of such encounters.

The virulence of an introduced parasite, hereafter defined as the degree of the effect on host reproductive success, is often determined by attributes of both the parasite and the infected host (Toft, 1991). Island populations of hosts are particularly susceptible to the effects of introduced parasites (Reid & Miller, 1989). Restricted dispersal, low genetic diversity, and inbreeding depression can predispose island populations to extinction even in the absence of introduced parasites (Delannoy & Cruz, 1991, Frankham, 1998). These same characteristics limit variation in available host defense mechanisms, which can further relax selection on parasite virulence. Parasites with low host specificity and high dispersal can become quite virulent. The availability of alternative host populations or species means that introduced parasite populations can remain stable even if a given host population is driven to extinction (de Castro & Bolker, 2004).

The recent introduction of a nest ectoparasite, *Philornis downsi*, to the Galapagos Islands, presents a rare opportunity to study the initial interactions of a novel host-parasite association. *P. downsi* was originally described from Trinidad and Brazil (Dodge & Aitken, 1968, Couri, 1985), and was introduced to the Galapagos as early as the 1960's. However, *P. downsi* was first observed in the nests of Darwin's finches in 1997. It is since been documented on 11 of 13 major islands in the Galapagos archipelago and in the nests of at least 14 species of birds, including 9 species of

Darwin's finches (Fessl & Tebbich, 2002, Wiedenfeld et al., 2007, Fessl et al., 2010, O'Connor et al., 2009). *P. downsi* have already been implicated in the severe decline of several Darwin's finch species (O'Connor et al., 2009, Grant et al., 2005).

My dissertation examines the interactions between *P. downsi* and a relatively abundant species of Darwin's finch, the medium ground finch (*Geospiza fortis*). I use an experimental approach to investigate the effects of *P. downsi* on medium ground finch reproductive fitness (Chapter 2). Then, I investigate whether medium ground finches can mount parasite specific antibody-mediated immune responses to *P. downsi* and avian poxvirus, a pathogen that is also present in some populations of Darwin's finches (Chapter 3). I further validate the use of an immuno-assay using house sparrow antiserum to detect *P. downsi*-specific antibodies in seven species of Darwin's finches parasitized by *P. downsi* (Chapter 4). I then examine whether medium ground finch immunological and behavioral defense mechanisms are effective against *P. downsi* (Chapter 5). Finally, I use a population viability model to predict the persistence of medium ground finch populations in light of the observed effects of *P. downsi* parasitism on host survival (Chapter 6).

Chapter Summaries

Chapter 2: Experimental demonstration of the fitness consequences of an introduced parasite of Darwin's finches

Chapter 2 investigates the effects of *Philornis downsi* on the fitness of medium ground finches. Several studies report that *P. downsi*, recently introduced to the Galápagos Islands, reduces fitness of its avian hosts. However, most of these studies are based on correlational or observational data. A single previous experimental study was

performed but with small sample sizes that required the authors to combine results across species (Fessl et al., 2006). While these studies were integral in bringing attention to the potential impact of this introduced parasite on native birds, a more rigorous experimental manipulation was needed to measure the direct effect of the parasite on host fitness. We performed a large-scale experimental study using nest liners to manipulate parasite abundance in the nests of medium ground finches. We quantified the impact of the parasite on nestling growth and fledging success. Nest liners significantly reduced, but did not completely eliminate *P. downsi* in nests. A reduction in parasite abundance resulted in a significant increase in the number of nests that successfully fledged young. Nestlings in parasite-reduced nests also tended to be larger prior to fledging. By using an experimental approach, our results confirm that *P. downsi* has significant negative effects on the fitness of medium ground finches. Furthermore, our results showed that a reduction in parasite load is sufficient to significantly increase fledging success, information that may be useful in the design of management plans for controlling *P. downsi* populations.

Chapter 3: Ecoimmunity in Darwin's finches: invasive parasites trigger acquired immunity in the medium ground finch (*Geospiza fortis*)

In Chapter 3, we investigate host immune responses against two classes of parasites, the ectoparasitic nest fly, *Philornis downsi*, and pox virus (*Poxvirus avium*). We developed an enzyme linked immunosorbent assay (ELISA) using house sparrow antiserum to test for the presence of parasite specific antibodies in the serum of medium ground finches. Finches from populations affected by pox had higher pox-specific antibody responses than finches from populations without visible symptoms of the virus.

Finches had higher *Philornis*-specific antibody responses during the breeding season, when exposure to the nest fly occurs, compared to finches prior to the breeding season. Female medium ground finches had higher *Philornis*-specific responses than males, consistent with increased exposure while females brood nestlings (males do not brood). This study was one of the first to show parasite-specific antibody responses to multiple classes (intracellular and ectoparasitic) of parasites in a wild population of avian hosts. Development of a parasite specific immuno-assay is the first step in determining whether Darwin's finches are able to defend themselves immunologically against introduced parasites.

Chapter 4: Test for parasite-specific immune response in multiple species of Darwin's finches

Chapter 4 validates the use of an immuno-assay to detect parasite specific antibodies in multiple species of Darwin's finches. We used house sparrow antiserum (*Passer domesticus*) to develop an enzyme-linked immunosorbent assay (ELISA) that detects parasite-specific antibodies in the serum of medium ground finches (*Geospiza fortis*) (Chapter 3). Here, we test whether this same technique can be used with serum from other species of Darwin's finches. We compared cross-reactivity of serum from seven species of Darwin's finches with antiserum from house sparrows using a total-IgY sandwich ELISA and tests of dilutional parallelism. Our results show that house sparrow antiserum cross-reacts well with serum from seven other species of Darwin's finches. We then tested whether these same seven host species produced parasite-specific antibodies against the introduced parasitic fly, *Philornis downsi*. All seven species are known hosts of this parasite and our results show that all seven species

produced *P. downsi*-specific antibodies. This is the first study to demonstrate a parasite-specific antibody response in a group of closely related wild host species. Validation of this technique and confirmation of the presence of *P. downsi*-specific antibodies in multiple species of Darwin's finches provides the necessary framework for comparative studies of immune defense against an introduced parasite.

Chapter 5: Are Darwin's finches sitting ducks? Ineffective host defenses against an introduced parasite

Chapter 5 investigates the presence and efficacy of immunological and behavioral defenses of the medium ground finch against the introduced parasite, *Philornis downsi*. Hosts can use a variety of defense mechanisms to mitigate the negative effects of parasitism. However, host populations that encounter introduced parasites may not yet have effective defense mechanisms. *P. downsi* is a hematophagous nest parasite recently introduced to the Galapagos Islands where it infests the nests of multiple species of land birds, including Darwin's finches. *P. downsi* negatively impacts nestling growth and fledging success, posing a serious threat to the reproductive fitness of its hosts. The goal of this study was to investigate whether medium ground finches possess defense mechanisms against *P. downsi* that are effective in mitigating at least some of the negative effects of this parasite. We used a fumigant to eliminate *P. downsi* from the nests of medium ground finches and monitored nestling growth and fledging success in fumigated and control nests. We used nest cameras to record parental and nestling behaviors during the day and nighttime and quantified *P. downsi*-specific antibody responses in parent and nestling finches. We found no evidence of effective behavioral defenses by parent or nestling finches, though observed

changes in behavior helped elucidate possible mechanisms by which *P. downsi* causes nestling mortality. Nestlings did not produce *P. downsi*-specific antibodies, nor were maternally transferred antibodies present when nestlings were five days old. Adult females in parasitized nests had a significantly stronger *P. downsi*-specific antibody response than females in unparasitized nests. Among females in parasitized nests, there was a weak correlation suggesting that greater adult female *P. downsi*-specific antibody responses decreased parasite abundance in nests. While all fumigated nests fledged at least one offspring, all control nests had complete nest failure (100% mortality). This results suggests that none of the observed behavioral or immunological responses to *P. downsi* were effective, at least during our study.

Chapter 6: The demise of Darwin's finches? A modeling approach to assess the impact of an introduced parasite on host population viability

In Chapter 6 we use a population viability model to predict the persistence of medium ground finches affected by the nest parasite, *Philornis downsi*. Introduced parasites and pathogens present one of the greatest threats to naïve host populations, especially those on islands. *P. downsi* has already been implicated in the severe population declines of several endangered Darwin's finch species. We develop a model largely based on data from our own three-year experimental study of the effects of *P. downsi* on medium ground finch reproductive fitness. The model predicts that extinction of medium ground finches on the island of Santa Cruz is likely within the next half-century, demonstrating the devastating impact *P. downsi* can have on even relatively large populations of finches. We use the predictions of our model to highlight the need for additional experimental research on the effects of *P. downsi* on other populations and

species of finches. By manipulating various parameters of the model we show the extent to which *P. downsi* prevalence needs to be reduced to increase the predicted time to host extinction beyond 100 years. The predictions of our model are meant to serve as a warning of the potential impact of this fly on Darwin's finches. We discuss conservation efforts currently underway to control *P. downsi* populations and hope that the predictions of our model reinforce the need for such intervention.

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CHAPTER 2

EXPERIMENTAL DEMONSTRATION OF THE FITNESS CONSEQUENCES OF AN INTRODUCED PARASITE OF DARWIN'S FINCHES

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Experimental Demonstration of the Fitness Consequences of an Introduced Parasite of Darwin's Finches

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Abstract

Background: Introduced parasites are a particular threat to small populations of hosts living on islands because extinction can occur before hosts have a chance to evolve effective defenses. An experimental approach in which parasite abundance is manipulated in the field can be the most informative means of assessing a parasite's impact on the host. The parasitic fly *Philornis downsi*, recently introduced to the Galápagos Islands, feeds on nestling Darwin's finches and other land birds. Several correlational studies, and one experimental study of mixed species over several years, reported that the flies reduce host fitness. Here we report the results of a larger scale experimental study of a single species at a single site over a single breeding season.

Methodology/Principal Findings: We manipulated the abundance of flies in the nests of medium ground finches *(Geospiza fortis)* and quantified the impact of the parasites on nestling growth and fledging success. We used nylon nest liners to reduce the number of parasites in 24 nests, leaving another 24 nests as controls. A significant reduction in mean parasite abundance led to a significant increase in the number of nests that successfully fledged young. Nestlings in parasite-reduced nests also tended to be larger prior to fledging.

Conclusions/Significance: Our results confirm that *P. downsi* has significant negative effects on the fitness of medium ground finches, and they may pose a serious threat to other species of Darwin's finches. These data can help in the design of management plans for controlling *P. downsi* in Darwin's finch breeding populations.

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Introduction

Introduced parasites and pathogens are an increasing problem as economic growth and trade provide further opportunities for species to invade [1]. Small, endemic populations of hosts, such as those on islands, are particularly at risk from introduced parasites and pathogens because extinction can occur before hosts have a chance to evolve effective defenses [2,3]. For example, the introductions of avian malaria and its mosquito vector to the Hawaiian Islands have been implicated in the rapid extinction of several endemic honeycreeper species [4,5,6]. The Galápagos Islands have fared better; none of the birds endemic to this archipelago have suffered extinction due to parasites or pathogens over recorded history [7]. However, recent pressure from introduced parasites and pathogens has the potential to cause serious population declines, if not extinctions [8,9].

A parasite of particular concern is the recently introduced fly, *Philornis downsi* (Diptera: Muscidae; Dodge & Aitken) [10]. To our knowledge, there are no studies of the fitness consequences of *P. downsi* on hosts within the native range of this fly. Aside from the Galapagos, the only other records of *P. downsi* are from Trinidad and Brazil [11]. *P. downsi* was not observed in the nests of birds in the Galapagos until 1997 [12]. *P. downsi* is now known to parasitize at least 14 species of Galápagos land birds, including 9 species of Darwin's finches [12,13,14]. It has been found on 11 of the 13 Galápagos Islands sampled [15]. *P. downsi* may be partly responsible for recent declines of the endangered mangrove finch (*Camarhynchus heliobates*), the endangered medium tree finch (*Camarhynchus pauper*), and the warbler finch (*Certhidea fusca*) [8,9,13].

P. downsi is an obligate nest parasite of birds. While the adult flies are non-parasitic (they feed on decaying matter), the larvae are semi-hematophagous parasites of nestlings [16] (Fig. 1A). *P. downsi* larvae chew through the skin of nestlings and consume blood and other fluids [16] (Fig. 1B). Larvae feed primarily at night; during the day most larvae burrow into the nest material [17]. Adult flies lay their eggs in the nesting material and nares (nostrils) of nestlings [18,19]. After the eggs hatch, the larvae complete three instars, the first of which can live in the nares of the

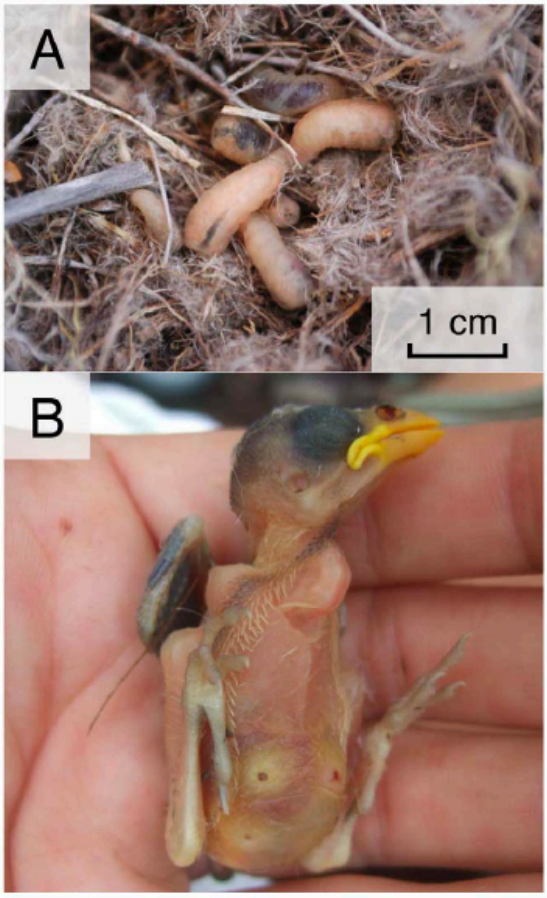

Figure 1. Study organisms. A) *Philornis downsi* larvae in the nest of a medium ground finch *(Geospiza fortis)*; photo courtesy of A. Hendry; B) *G. fortis* nestling with three lesions on the abdomen and damage to the nares (nostrils) from *P. downsi* larvae.
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host or freely in the nest material. Damage to the nares of nestlings can persist into adulthood [20]. Second and third instar larvae live freely in the nest material, where they eventually pupate and later emerge as adult flies.

Earlier studies of the impact of *P. downsi* on Darwin's finches identified this parasite as a potential threat (Table 1). Several studies report a negative correlation between *P. downsi* abundance and fledging success [21,22,23,24]. Additional studies report varying degrees of nest failure (complete or partial brood loss) based on finding *P. downsi* in nests [12,13,14,19]. While these studies have been integral in bringing attention to the impact of *P. downsi* on various finch species, the next step is to measure the direct effect of the parasite, while controlling for other variables that may be contributing to nest failure (e.g. ecological variables such as rainfall and food availability, which differ from year to year [25,26]).

To measure the magnitude of a parasite's direct effect on a host, an experimental approach is necessary [27,28]. Correlations between parasite abundance and host fitness can be difficult to interpret because they do not measure the direct effect on host fitness. For example, poorly fed birds can have high numbers of parasites because they have little energy to invest in defense, while also having low reproductive success because they have little energy to invest in offspring. The consequence is a spurious correlation (or at least an inflated one) between parasite abundance and host fitness.

To date, just one published study has experimentally manipulated *P. downsi* abundance and measured its impact on Darwin's finches. Fessl *et al.* [29] eliminated *P. downsi* from four *Geospiza fortis* nests, and eight *G. fuliginosa* nests, by fumigating the nests with a 1% pyrethrin solution. Following treatment, the authors monitored nestling growth over a four-day period; they also monitored nestling hemoglobin level and the fledging success of each nest, compared to non-fumigated nests. Though limited sample sizes required them to pool data between species and across years, their results showed that nestlings in fumigated nests tended to have higher hemoglobin concentrations, a significantly higher growth rate, and significantly greater fledging success than nestlings in non-fumigated nests (Table 1).

Here we report the results of a larger scale experimental study of a single species of Darwin's finch at a single site over a single breeding season. We manipulated the abundance of flies in the nests of medium ground finches *(Geospiza fortis)* and quantified the impact of the parasites on nestling growth and fledging success.

Materials and Methods

Ethics statement

All procedures were approved by the University of Utah Institutional Animal Care and Use Committee (protocol #07-08004).

Study site and experimental design

Our study was conducted January-April, 2008 at El Garrapatero on Santa Cruz Island in the Galápagos Archipelago, Ecuador. *G. fortis* is abundant at this site [23], where it builds nests in endemic tree cacti (*Opuntia echios gigantea*) and *Acacia* trees, 1.5 to 4 meters above the ground. Clutch size ranges from 2–5 eggs. The incubation period is approximately 12 days, and nestlings spend 10–14 days in the nest prior to fledging. Both sexes of *G. fortis* feed nestlings and clean the nest, but only females incubate eggs and brood hatched offspring. Breeding pairs of adults often re-nest, but they do not use the same nest again [26].

We searched a 1.5 km×1.5 km area for active *G. fortis* nests throughout the breeding season. We monitored a total of 48 nests, all of them constructed in tree cacti, by 34 different breeding pairs of finches. Fourteen (29%) of the nests in our sample were repeat bouts of nesting during the study period. Adult birds were netted near the nest and fitted with a numbered Monel metal band and three plastic color bands for identification at a distance. Active nests were visited every other day between the hours of 0600 and 1100, and the number of eggs and nestlings were recorded. Nests were included in the experiment if they were discovered before the eggs hatched (n = 44 nests) or, in the case of four nests, soon after hatching (nestlings ≤5 days of age, but these four nests were omitted from all analyses of growth). We continued to check nests and process nestlings (see below) until the oldest nestling was 10 days of age, or until all of the nestlings died. Processing nestlings older than 10 days of age can trigger premature fledging [30]. Therefore, once the oldest nestling reached 10 days of age, we stop processing nestlings. *G. fortis* nests have a side entrance that makes it possible to census older nestlings from a distance with binoculars. Once empty, nests were collected to count parasites.

Nests were randomly assigned to the experimental group (n = 24 nests) or control group (n = 24 nests). In most cases of re-nesting by

Table 1. Tests of the impact of *Philornis downsi* on Darwin's Finches.

Darwin's Finch Species	Nestling Hb Level	Nestling Growth	Fledging Success			Reference
			Obs*	Cor†	Exp‡	
Geospiza fortis	-	N	-	Y	-	[23]
Geospiza fuliginosa[α]	Y	-	-	Y	-	[21]
Geospiza fuliginosa	-	-	-	Y	-	[24]
G. fortis & fuliginosa[β]	Y	Y	-	-	Y	[29]
G. fortis, fuliginosa & scandens[β]	-	-	Y	-	-	[19]
Camarhynchus pauper	-	-	Y	-	-	[13]
Camarhynchus heliobates	-	-	Y	-	-	[14]
4 species (3 genera)[β,Ω]	-	-	Y	-	-	[12]
6 species (4 genera)[β,θ]	-	-	-	Y	-	[22]

(Y, impact of parasite on host parameter detected; N, no impact detected; -, not tested).
*Observational data suggest *P. downsi* responsible for nestling mortality.
†Correlational data show a negative relationship between parasite abundance and fledging success.
‡Experimental nests fumigated to reduce parasite abundance.
[α]Different islands pooled for analysis.
[β]Different species pooled for analysis.
[Ω]Geospiza fuliginosa, Camarhynchus parvulus, Cam. psittacula, Certhidea olivacea.
[θ]Geospiza fuliginosa, G. fortis, Camarhynchus parvulus, Cam. psittacula, Cactospiza pallida, Certhidea olivacea.
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a single pair of birds, the treatment was reversed between reproductive bouts. The floors of experimental nests were fitted with a liner constructed from a small section of nylon stocking stretched over a wire hoop (~9 cm in diameter). The liner prevented most of the fly larvae in the bottom of the nest from reaching the nestlings. This approach has been effective in other experimental manipulations of nest parasites [31]. Experimental nests were fitted with liners within one day of the first egg hatching (a clutch of eggs normally hatches over two to four days). The four nests that already contained nestlings when first monitored were all assigned to the unlined group because they could have already been exposed to parasites. Parasite larvae occasionally crawled over the liners, coming into contact with nestlings. For this reason, liners were carefully examined and cleaned or replaced each time the nests were checked. Any larvae found and removed were included in final counts of parasite abundance, since these parasites may have been able to feed on nestlings and may have affected nestling growth and survival.

Nestling growth

At each nest check the nestlings were weighed with a digital balance (Ohaus, 0.1 g accuracy). In addition, the following measurements were taken with digital calipers (Fisherbrand, 0.01 mm accuracy): bill length, bill depth, bill width, tarsus length, and length of the outermost primary feather from where it emerged from the skin to its distal tip. At the first visit after hatching, nestlings were aged based on body mass using data from Boag [32], as follows: ≤1.9 grams (1 day old); 2–2.9 grams (2 days old); 3–3.9 grams (3 days old). New nestlings were marked individually by coloring a toenail with a permanent marker. At three to four days of age they were given a single plastic color band. When nestlings were at least seven days of age they were fitted with a numbered Monel metal band and three plastic color bands.

Because Darwin's finches have asynchronous hatching, the fact that we processed nests on alternate days meant some birds ("odd day birds") were processed for the first time at one day of age - and on odd days thereafter - until they were nine days old. Other birds ("even day birds") were processed for the first time at two days of age - and on even days thereafter - until they were ten days old. These two data sets were used to construct growth curves for lined and unlined treatments.

Fledging success

Fledging was confirmed by observing and identifying birds on the basis of their color bands after they left the nest.

Parasite abundance

After each nesting bout we removed the nest and placed it in a sealed plastic bag. The nest was carefully dissected within eight hours of collection and *P. downsi* larvae, pupae, and eclosed pupal cases were counted. First instar larvae, which are too small to discern reliably in the nest material, were not included in counts of parasite abundance. Total parasite abundance was the sum of second and third instar larvae, pupae, and eclosed pupal cases. Other types of fly larvae, e.g. Sarcophagidae, were identified but not included in counts of total parasite abundance because these larvae are not parasitic; they feed on the tissues of dead nestlings [29].

Statistical analyses

Statistical analyses were done in Prism® v.5.0b (GraphPad Software, Inc.) and R v.2.12.2 (R Development Core Team). Nestling growth was analyzed using regressions and two-tailed t-tests. For some growth parameters we also calculated effect size, i.e. the mean difference in a growth parameter between the lined and unlined treatments [33]. We used bootstrapping (10,000 repetitions) to construct 95% confidence intervals around mean effect sizes [33].

It was not possible to analyze growth over time using repeated measures ANOVA or GLMM because extensive mortality in one of the groups (>80% prior to fledging in unlined, heavily parasitized nests) made sample sizes very uneven over time. Therefore, growth data were tested for an effect of treatment simply by comparing the final values taken for lined nests and unlined nests, when nestlings were nine or ten days old. Thirteen

nestlings in seven unlined nests survived to at least nine days of age compared to 26 nestlings in twelve lined nests. To avoid pseudoreplication, we used the mean brood value of nine and ten day old nestlings in each nest. The data for nine and ten day old birds were combined for analysis unless there was an effect of age on the growth parameter of interest (determined via regression analysis). There was an effect of age only in the case of outermost primary feather length, which still had not begun to asymptote by Days 9 and 10 ($R^2 = 0.30$, $p = 0.003$). Therefore, the feather data were analyzed separately for nests containing nine and ten day old nestlings.

Results

Parasite abundance

P. downsi was present in 43 of 48 *G. fortis* nests (90%). Liners presumably did not prevent adult flies from laying eggs in nests; however, if liners reduced the number of opportunities for larvae to feed, then lined nests should have had fewer parasites than unlined nests. In support of this prediction, we found that lined nests had significantly fewer parasites per nest than unlined nests (mean parasite load ± SE = 21.79±3.56 in lined nests, compared to 37.50±4.92 in unlined nests; Welch's t-test, $t = 2.58$, $df = 41$, $p = 0.01$ (Fig. 2)).

Nestling growth

Nestlings in lined nests were not significantly heavier than nestlings in unlined nests ($t = 1.73$, $df = 18$, $p = 0.10$; Fig. 3A). However, an analysis of effect size revealed that nestlings in lined nests (mean ± SE, 12.7±0.4 g) were 1.7 g heavier, on average, than nestlings in unlined nests (11.0±1.0 g), with a 95% CI = −0.3 g to 3.7 g. Thus, nestlings in lined nests could range from 3.7 g heavier than nestlings in unlined nests, to 0.3 g lighter; however, they were lighter in only 5% of the bootstrap samples.

Tarsus length did not differ significantly between nestlings in lined (18.14±0.34 mm) versus unlined nests (17.23±0.45 mm) ($t = 1.64$, $df = 18$, $p = 0.12$; Fig. 3B). However, analysis of effect size showed that nestlings in lined nests had tarsi 0.91 mm longer than nestlings in unlined nests (95% confidence interval = −0.09 mm to 1.97 mm). The 95% CI around this effect size indicated that nestlings in lined nests could have tarsi up to

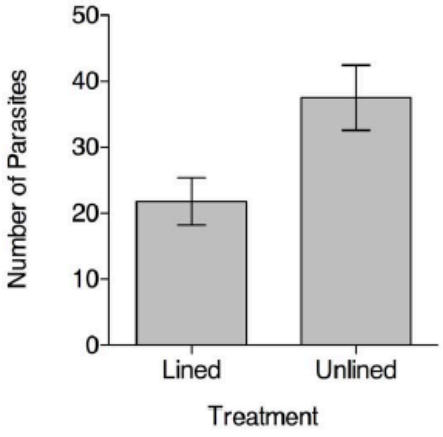

Figure 2. Comparison of the mean (±SE) number of *P. downsi* in lined and unlined nests.
doi:10.1371/journal.pone.0019706.g002

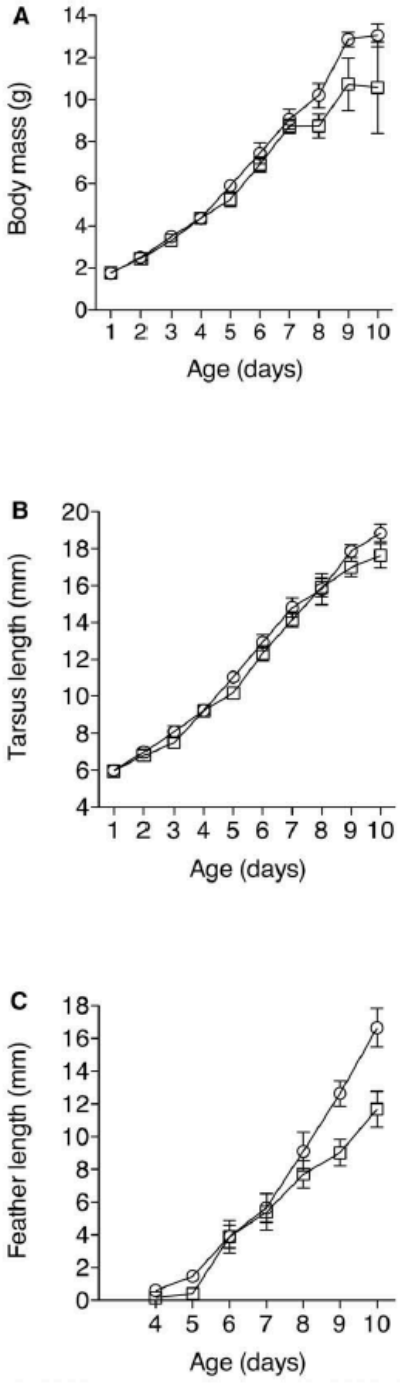

Figure 3. Comparison of mean (±SE) growth parameters for nestlings in lined (◯) and unlined (□) nests, including body mass (A), tarsus length (B), and outermost primary feather length (C).
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1.97 mm longer, on average, than nestlings in unlined nests. Alternatively, nestlings in lined nests could have tarsi up to 0.09 mm shorter than nestlings in unlined nests, but only in 4% of the bootstrap samples.

Outermost primary feathers of "odd day" nestlings in lined nests (12.64±0.77 mm) were significantly longer than those of nestlings in unlined nests (9.02±0.82 mm) ($t = 3.13$, $df = 13$, $p = 0.008$; Fig. 3C). Outermost primary feathers of "even day" nestlings in lined nests (16.65±1.18 mm) were also significantly longer than those of nestlings in unlined nests (11.67±1.10 mm) ($t = 2.27$, $df = 10$, $p = 0.05$).

A composite measure of bill size, using a principal components analysis of bill length, bill width, and bill depth [26], revealed that PC1 explained 68.5% of the variation (eigenvalue = 2.05). However, PC1 did not differ significantly between nestlings in lined and unlined nests ($t = 0.831$, $df = 18$, $p = 0.42$), nor was there a strong trend.

Fledging success

Nestlings in lined nests had significantly greater fledging success than nestlings in unlined nests. Eight of 24 lined nests (33%) fledged young, compared to just one of 24 (4%) unlined nests (Fisher's exact test, $p = 0.02$, Fig. 4A). We also compared the number of individual nestlings that fledged from lined versus unlined nests: 19 of 75 nestlings (25%) from lined nests successfully fledged, compared to only three of 67 nestlings (4%) from unlined nests ($p<0.001$; Fig. 4B). Thus, the experimental reduction in parasite number had a clear positive impact on fledging success.

Discussion

Our study is a rigorous experimental test of the impact of *P. downsi* on the fitness of Darwin's finches. Our experimental design minimized variation between species, sites and years, allowing us to quantify the direct effect of *P. downsi* on parameters of host fitness. We manipulated parasite abundance in a relatively large number of medium ground finch nests using nest liners, rather than chemical fumigants, thus eliminating any possible side effects of pesticides on nestling growth or other fitness components [34]. Liners reduced parasite abundance by 42%, on average. This reduction in parasite load led to a significant increase in the number of nests that successfully fledged young. Our results are consistent with those of Fessl *et al*. [29], who also found a significant increase in the number of nests that successfully fledged young when parasites were completely eliminated through the use of a fumigant.

Our study further suggests that *P. downsi* has a negative effect on nestling growth. When we tested the impact of experimental treatment on nestling size using outermost primary feather length as an index of growth, there was a clear difference. Nestlings in unlined nests had outermost primary feathers that were 30% shorter than nestlings in lined nests, indicating that birds fledging from unlined nests would have underdeveloped feathers. Feather length is a sensitive measure of growth in birds, because feathers grow more rapidly than overall body mass or tarsus length [32,35,36].

Nestlings in unlined nests also tended to have lower body mass, and shorter tarsi, than nestlings in lined nests. The effect of *P. downsi* on nestling mass and tarsus length are consistent with other studies testing for effects of parasitic flies on nestling growth. In our study, nestlings in unlined nests weighed a mean of 13% less, and had tarsi that were a mean of 5% shorter than nestlings in lined nests. In comparison, nestling Blue tits (*Parus caeruleus*) and House wrens (*Troglodytes aedon*) parasitized by blowflies (*Protocalliphora*) weighed 3–6% less and had tarsi 0–2% shorter than unparasitized nestlings, prior to fledging [37,38].

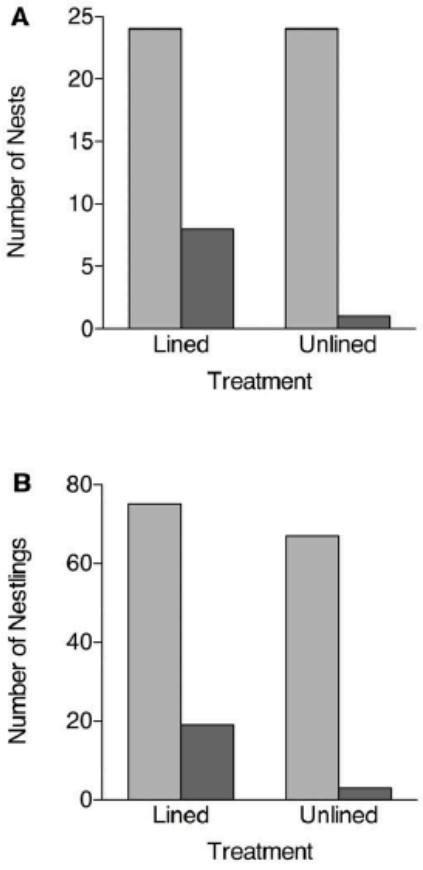

Figure 4. Effect of liners on host fledging success. Light bars are the total number of (A) nests and (B) nestlings monitored. Darker bars are (A) the number of nests that fledged one or more young, and (B) the total number of fledglings from nests in each treatment.
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Our data show that experimentally reducing parasite abundance leads to a reduction in nestling body mass, tarsus length, and outermost primary feather length. Only the reduction in feather length was statistically significant; however, the fact that the effects on body mass and tarsus length were large in size, and in the same direction as the effect on feather size, suggests that *P. downsi* does, in fact, reduce nestling growth.

Our data showed no effect of parasitism on the bill sizes of nestlings, as estimated by a principal component analysis. However, the bill length, width and depth of *Geospiza* finches are known to increase more slowly than body mass, tarsus and wing chord [32]. Morphological traits such as flight feathers must grow quickly in order for nestlings to be capable of flying soon after they leave the nest. Similarly, nestlings with high body mass are more likely to survive after fledging than nestlings with low body mass [39]. *Geospiza* adults use their bills to crack seeds for food; however, seed cracking ability is not as important in young fledglings because adults continue feeding them after they leave nest [32].

Body size at fledging is known to predict post-fledging survival in birds [39,40]. Therefore, it is likely that even a small effect of parasitism on nestling size prior to fledging will place birds at a

significant disadvantage. Although we did not monitor post-fledging survival in our study, it is possible that fledglings from our unlined nests did not survive as well as the larger fledglings from lined nests. Thus, the impact of *P. downsi* on host reproductive success may have extended beyond the demonstrated impact on fledging success. Further study is needed to monitor post-fledging success in order to more fully understand long-term effects of *P. downsi* parasitism, in addition to the more immediate impact of the parasites on growth and fledging success.

While we did not test the effect of treatment on growth parameters repeatedly over the developmental period of the nestlings, the differences in growth were not apparent until nestlings were older in any case (Fig. 3A–C). The late appearance of growth differences between nestlings in lined and unlined nests may have been a byproduct of our method of parasite manipulation. *P. downsi* eggs and first instar larvae are often found in the nares (nostrils) of nestlings [19]. For this reason, the use of nylon liners would not necessarily affect the first instar stage of the parasite. It is possible that young nestlings in both lined and unlined nests experienced similar levels of first instar parasitism and, thus, similar effects on growth at an early age. In contrast, nest liners inhibited second and third instar larvae, which spend most of their time in the nest material. Thus, the impact on nestling size reported in our study may have been due primarily to second and third instar larvae.

P. downsi parasitism may affect nestlings through several non-mutually exclusive mechanisms. Blood-feeding parasites can lower hemoglobin concentrations in nestlings, causing anemia [41,42]. Dudaniec *et al.* [21] found a negative correlation between *P. downsi* abundance and hemoglobin concentration in small ground finches (*G. fuliginosa*, Table 1). Fessl *et al.* [29] found that nestlings from parasitized nests tended to have lower hemoglobin concentrations than nestlings in unparasitized nests. Although we did not measure hemoglobin concentration in this study, our more recent work confirms that nestlings in parasitized nests have lower hematocrit (total red blood cell volume) than nestlings in unparasitized nests (Koop, unpublished data).

P. downsi may also affect nestling behavior and impede condition signaling to parents. Nestlings that are weakened by parasites may not have enough energy to beg for food [43]. Nestling begging intensity is correlated with the amount of food parents provide in other species of birds [44]. Even if nestlings are fed adequately, those in parasitized nests may suffer energetic costs that eventually lead to decreased survival. A recent study by O' Connor *et al.* [17] reported avoidance behaviors by nestling Darwin's finches toward *P. downsi* larvae in the nest. Larvae were most active at night; nestlings kept awake at night by feeding larvae presumably have less energy for growth. *P. downsi* larvae may also affect nestling growth indirectly by affecting parental behavior. Adult females irritated by feeding larvae, or by restless nestlings, may choose to stop brooding young, decrease feeding visits to the nest, or abandon the nest entirely. Further study is needed to investigate the proximal mechanisms underlying costs of *P. downsi* parasitism on fledging success.

Our study further demonstrates the devastating effect that *P. downsi* has on host fledging success. Only a single nest from the unlined treatment produced fledglings that were sighted after leaving the nest. A 42% experimental reduction in parasite abundance was sufficient to significantly increase the number of nests that fledged young. Thus, conservation efforts aimed at controlling *P. downsi* may be effective even if fly populations are simply reduced but not necessarily eliminated. Future monitoring is needed to determine whether the impact of *P. downsi* on nesting finches scales up to the level of populations and species [45,46]. There is still much to learn about the ecology of *P. downsi* both in its native and introduced geographic ranges.

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Author Contributions

Conceived and designed the experiments: JAHK SKH DHC. Performed the experiments: JAHK SKH DHC. Analyzed the data: JAHK SKH SML DHC. Contributed reagents/materials/analysis tools: DHC. Wrote the paper: JAHK SKH SML DHC.

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CHAPTER 3

ECOIMMUNITY IN DARWIN'S FINCHES: INVASIVE PARASITES TRIGGER ACQUIRED IMMUNITY IN THE MEDIUM GROUND FINCH (*GEOSPIZA FORTIS*)

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Ecoimmunity in Darwin's Finches: Invasive Parasites Trigger Acquired Immunity in the Medium Ground Finch (*Geospiza fortis*)

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Abstract

Background: Invasive parasites are a major threat to island populations of animals. Darwin's finches of the Galápagos Islands are under attack by introduced pox virus (*Poxvirus avium*) and nest flies (*Philornis downsi*). We developed assays for parasite-specific antibody responses in Darwin's finches (*Geospiza fortis*), to test for relationships between adaptive immune responses to novel parasites and spatial-temporal variation in the occurrence of parasite pressure among *G. fortis* populations.

Methodology/Principal Findings: We developed enzyme-linked immunosorbent assays (ELISAs) for the presence of antibodies in the serum of Darwin's finches specific to pox virus or *Philornis* proteins. We compared antibody levels between bird populations with and without evidence of pox infection (visible lesions), and among birds sampled before nesting (prior to nest-fly exposure) versus during nesting (with fly exposure). Birds from the Pox-positive population had higher levels of pox-binding antibodies. *Philornis*-binding antibody levels were higher in birds sampled during nesting. Female birds, which occupy the nest, had higher *Philornis*-binding antibody levels than males. The study was limited by an inability to confirm pox exposure independent of obvious lesions. However, the lasting effects of pox infection (e.g., scarring and lost digits) were expected to be reliable indicators of prior pox infection.

Conclusions/Significance: This is the first demonstration, to our knowledge, of parasite-specific antibody responses to multiple classes of parasites in a wild population of birds. Darwin's finches initiated acquired immune responses to novel parasites. Our study has vital implications for invasion biology and ecological immunology. The adaptive immune response of Darwin's finches may help combat the negative effects of parasitism. Alternatively, the physiological cost of mounting such a response could outweigh any benefits, accelerating population decline. Tests of the fitness implications of parasite-specific immune responses in Darwin's finches are urgently needed.

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Introduction

Invasive parasites pose a serious threat to native animal populations, because hosts with no history of exposure may lack effective immune defenses. Invasive parasites are a particular threat to small, island populations [1,2]. For example, introduced malaria (*Plasmodium relictum*) has exacerbated the decline of Hawaiian honeycreeper species, many of which are now extinct [3,4]. Darwin's finches have recently been exposed to two introduced parasites of high conservation priority: avian pox virus (*Poxvirus avium*) and the nest fly *Philornis downsi* (Figure 1A, 1B) [1,2]. Both of these parasites have been shown to have negative effects on host fitness of Galápagos birds [5,6,7,8,9,10]. If birds are able to mount an immune response to these novel pathogens, then they might ultimately be protected, to at least some degree, from the negative fitness consequences of parasitism. Alternatively, the physiological costs of an induced immune response to these parasites may exceed the benefits of mitigating parasite damage and contribute to negative fitness consequences. Indeed, these contrasting possibilities are a guiding force behind research within the field of ecological immunology [11].

The prevalence of *Avipox* in the Galápagos Islands varies on a geographic scale. Over the past 35 years it has been absent or very rare at Daphne Major and El Garrapatero, Santa Cruz Island. Daphne Major had episodic outbreaks of pox in 1983 and 2008 [12], and during our study in 2008, we found 50% of birds to be

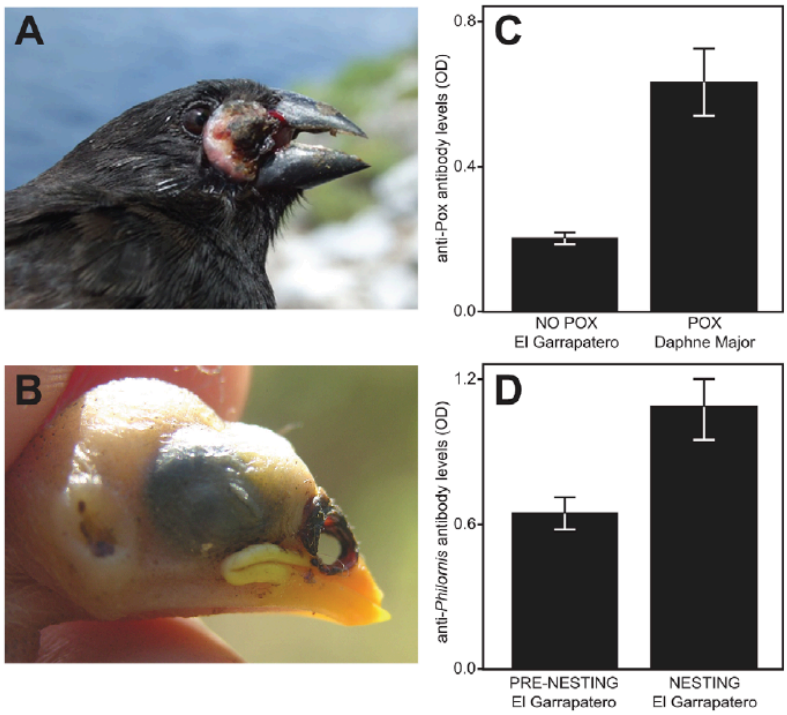

Figure 1. Parasite-specific antibody response of *Geospiza fortis*. (A) Medium ground finch, *Geospiza fortis*, with pox lesion in front of eye. (B) *G. fortis* nestling with *Philornis downsi* lesions in nostrils and ear. (C) Pox-binding antibody levels of adult birds on Daphne Major (n = 30) were higher than those of adult birds at El Garrapatero (n = 113) (Mann Whitney U = 619.50, $p<0.0001$). (D) *Philornis*-binding antibody levels of adult birds with active nests at El Garrapatero (n = 37) were higher than those of adult birds prior to nesting (n = 76) at the same site (U = 800, $p<0.0001$). Antibody response is measured as the optical density (OD) at 450nm. Bars indicate mean±standard error.
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symptomatic for pox (15 out of 30 birds had active lesions). The outbreak of pox on Daphne Major in 2008 was not seen at El Garrapaterro. In 2008 not a single bird at El Garrapaterro, out of 129 individuals captured, was symptomatic, and none of these birds showed evidence of prior pox infection (e.g., scars or missing digits). The differences in pox prevalence between these two localities, allowed us to examine how infection influences pox-specific antibody levels in two populations with relatively similar histories of pox exposure.

Philornis downsi was first detected in the Galápagos in 1964; however, presence of the fly went relatively unnoticed until the late 1990's when large numbers of larvae were discovered in the nests of Galápagos land birds, including Darwin's finches [13,14]. Adult flies are not parasitic, but larvae are obligate parasites that feed on the blood and tissues of nestling birds. Nestling Darwin's finches exposed to fly larvae have reduced survival and growth [8,9]. At El Garrapatero in 2008, 96% of 23 nests were infested with *P. downsi*.

Ecological immunologists are exploring potential fitness trade-offs between immune defense against parasites and the physiological demands of other life-history traits (e.g. growth and reproduction). Although parasites are treated as a selective force acting on the immune system, few studies within ecological immunology use parasite-specific assays of immune function [15]. Non-specific assays do not clarify interactions between the immune system and parasites [16,17]. As a result, non-specific assays do not directly test fitness effects of immunological variation in the context of parasite pressure. Here we take the first step in examining avian responses to introduced parasites directly, by demonstrating parasite-specific antibody responses to multiple classes of parasites in Darwin's finches. We developed assays for parasite-specific antibody responses in the medium ground finch (*Geospiza fortis*) (see Methods). Our goal was to test for relationships between adaptive immune responses to novel parasites and spatial-temporal variation in the occurrence of parasite pressure among *G. fortis* populations. Our results demonstrate that Darwin's finches produce antibodies against these invasive parasites, and that the immune responses are correlated with spatial-temporal variation in parasite pressure, both between finch populations, and between sexes. To our knowledge, this is the first time parasite-specific immune responses have been demonstrated relative to multiple classes of parasites in a wild population of birds.

Results

Adult birds on Daphne Major had significantly higher levels of pox-binding antibodies than birds from El Garrapatero (mean±SE for Daphne Major = 0.63±0.09 optical density (OD); mean±SE for El Garrapatero = 0.20±0.02 OD; Mann Whitney U = 619.50; $p<0.0001$; Figure 1C).

When we compared *Philornis*-specific antibody levels in adult birds sampled before nesting (prior to *Philornis* exposure) with a different set of individuals sampled during the nesting period, we found significantly greater levels of *Philornis*-specific antibodies during the nesting period (mean±SE for nesting = 1.08±0.12 OD;

mean±SE for pre-nesting = 0.64±0.07 OD; Mann Whitney U = 800.00; p<0.0001; Figure 1D).

We found no sex difference in pox-specific antibody levels (mean±SE for Daphne Major females = 0.61±0.12 OD; mean±SE for Daphne Major males = 0.67±0.18 OD; Mann Whitney U = 91.50, p = 0.71), suggesting equal exposure of males and females to pox virus.

In contrast, we found significantly higher *Philornis*-specific antibody levels in females compared to males (mean±SE for El Garrapatero females = 0.99±0.11; mean±SE for El Garrapatero males = 0.58±0.06; Mann Whitney U = 1018.00, p = 0.001). This result is consistent with adult females having increased exposure to *P. downsi* when they brood offspring (males do not brood).

Discussion

Higher levels of pox-binding and *Philornis*-binding antibodies in Darwin's finches exposed to these parasites confirms that these birds are capable of mounting parasite-specific adaptive immune responses to novel parasites. Importantly, these antibody responses are directed against parasites that represent distinct immunological demands (intracellular versus external), and which constitute a serious threat to Darwin's finches. From the perspective of vertebrate immunology, it is not unusual that *G. fortis* is able to develop antibodies against novel challenges. However, our data are unique in two respects. This study is the first demonstration, to our knowledge, of ectoparasite-specific antibodies in a wild bird population. This study is also the first demonstration of parasite-specific antibodies directed against two distinct classes of parasites (external and intracellular) in a wild bird population. Within the field of ecological immunology, these observations are important because they establish a definitive immunological link between actual parasites and an animal of ecological interest [16].

These data also raise intriguing questions about prevailing assumptions regarding the host-parasite interactions of *P. downsi*. We found no differences in the levels of pox-binding antibodies between male and female finches. This finding agrees with the known ecology of avipox virus, which is transmitted by mosquitoes, or through bird-bird contact [1,7], where no bias in transmission among the sexes would be expected. In contrast, we found significantly higher *Philornis*-specific antibody levels in females compared to males, which agrees with the expected bias of higher female exposure to *P. downsi* during female brooding on the nest. Thus, our data cast doubt on the assumption that adults are never bitten [18].

The prevailing notion that adults are not exposed to larval feeding is based primarily on two observations: (i) lesions from larval feeding have not been observed on captured adult females; and (ii) the scaly covering on the females legs is thought to prevent larvae from penetrating the female's skin. The absence of obvious lesions on females does not rule out the possibility that adult females are bitten. For example, fewer than half of the nestlings in our study had visible lesions associated with larvae feeding, even though nests were heavily parasitized and in many cases nestlings died (unpublished data). Second, while larvae likely could not penetrate the scales on female's legs, females might be vulnerable to larval feeding through their brood patch, which is completely devoid of a feather covering. Larvae may come into contact with the female's brood patch while she is sitting on nestlings, particularly when larvae are in the first or second instar and reside on the nestlings (e.g., in the nostrils or on the wing webbing) [18].

Although the immunological data indicate feeding attempts on females do occur, we are not suggesting this is evidence that adult finches are viable hosts for *P. downsi*. Blood feeding attempts on adult birds may consistently fail for a variety of physical and behavioral reasons. However, if feeding attempts by larvae are occurring, it is reasonable to expect adult females are exposed to *P. downsi* antigens that are stimulating an immune response. The ecological importance of this immune response depends on multiple unexplored factors. For example, antibody development by the female could confer a defensive advantage to offspring, if there is transfer of maternal antibodies to the chicks [19]. If females are exposed during the first clutch and produce antibodies, they might transfer these antibodies to the eggs of their second or third clutch. Alternatively, a stimulated antibody response in the female could produce a physiological demand that reduces energy available for foraging and subsequent breeding attempts in the season. A number of important immunological questions must be answered to address these possible ecological outcomes. For example, how quickly are antibodies produced and how long do they persist? Though anti-ectoparasite antibodies can be produced rapidly (1-week) and persist up to two months without stimulation [20,21], the dynamics of anti-*Philornis* antibodies remain to be determined. We are currently attempting to determine if maternal antibodies are transferred to *G. fortis* offspring, as well as the timing of primary and secondary immune responses to *P. downsi* by female finches through the breeding season.

A critical next step in understanding the relationship between parasite infection and antibody production is to examine how these factors affect fitness. The only fitness data available for the effects of pox on Darwin's finches underscore the need for a detailed study of survival in relation to antibody response. Observations of *G. fortis* on Daphne Major in 2009 found that 11 out of 14 birds with pox symptoms in 2008 survived to the next year, compared with 12 out of 19 birds without pox symptoms (Fisher's exact test: two-tailed p = 0.46). These data suggest pox might not have the same impact on Darwin's finches as it does on Galápagos Mocking birds [5,12,22,23]. However, long-term fitness effects estimated in relation to short-term measures of prevalence are inadequate for several reasons. First, we do not know the severity of pox infection for individuals in our study. We only know that some birds on Daphne Major were exposed, whereas birds at El Garrapatero were not exposed over the course of our study. Variation in the intensity of exposure is likely related to survival. Second, we do not know if birds that were unexposed to pox at the time of sampling continued to be parasite-free. Finally, survival may be confounded by sex, age, condition, and breeding status, among other variables. For example, males and females might have different physiological responses to these diseases or the costs of breeding might be greater in one sex than the other. For example, some evidence suggests that males with prior pox exposure might have decreased pairing success [7]. We emphasize the need for future studies that control for these factors and that experimentally test for the impact of parasite load and antibody production on fitness. For example, survival data for birds with controlled exposure to pox can be compared between individuals with low versus high levels of anti-pox antibodies; these data would allow us to test the extent to which antibody production might be protective. Conversely, survival data for birds that are known to be free of active pox infection can be compared between individuals with anti-pox antibodies and those without anti-pox antibodies; these data would allow us to test whether antibody production might be costly. Studies such as these should be a major focus of future research, for both pox and *Philornis*.

In summary, the assays presented here are valuable tools for exploring the ecological immunology of Darwin's finches, and in helping to determine the epidemiology of two critically important diseases threatening avifauna in the Galápagos archipelago. Broadly, we expect this approach can be applied to other research

systems as well, which will strengthen studies that have typically relied on non-specific measures of immune function [16].

Methods

Ethics Statement

All procedures were approved by the University of Utah Institutional Animal Care and Use Committee (protocol #07-08004).

Sample Collection

We studied birds at two sites in the Galápagos Islands: El Garrapaterro, Isla Santa Cruz, and Isla Daphne Major. Birds were sampled at El Garrapaterro from January–April 2008 and at Daphne Major on March 11, 2008. They were captured using mist nests, or Potter's traps, and each bird was individually marked with a combination of one aluminum ring and three darvic color bands. We noted whether birds had active pox lesions, or evidence of prior pox infection (e.g., missing digits). We then collected a small volume of blood by piercing the ulnar vein with a 27-gauge needle. Approximately 50 µl of blood was collected with a capillary tube and expelled into centrifuge tubes. Centrifuge tubes were stored on ice in the field (approximately 6 hours), then transported to the laboratory where they were centrifuged. The serum was then pipetted off the top and stored at −80°C.

At El Garrapatero, we made focal observations of individuals to determine pairing status and nest location. We checked nests every other day to determine egg laying date, clutch size, and hatch date. When nests were no longer active (nestlings were predated, fledged, or died), the nests were dissected to obtain fresh *Philornis downsi* larvae, which were placed in a centrifuge tube and stored at −80°C for future antigen extraction (see below).

Adults sampled at El Garrapatero were assigned to one of two groups: un-exposed or exposed. Un-exposed birds (n = 76) were individuals that 1) had a nest but were sampled prior to the hatching of their first brood, 2) females that did not have a brood patch (and thus were not breeding), or 3) unmated males that were sampled early in the breeding season. Exposed birds (n = 37) were those sampled while they had nestlings in the nest and had parasites present in the nest. No unexposed individuals were re-sampled during the nesting period, and no exposed individuals were sampled prior to the nesting period.

For birds sampled at Daphne Major and El Garrapatero the sex was determined based on plumage (black plumage for males and the presence of a brood patch for females) or by genotyping. Blood samples of individuals for which we could not determine sex (nonbreeding females and young males have identical plumage) were sent to Avian Biotech International (Tallahassee, FL) for genotyping via PCR. On Daphne Major we sampled 10 females and 20 males; at El Garrapatero we sampled 56 females and 57 males.

Comparisons of pox immune response were made between populations (Daphne Major versus El Garrapatero). We did not compare asymptomatic and symptomatic birds within populations because it was not possible to evaluate the timing of prior pox exposure from current symptoms alone. Asymptomatic individuals could have elevated antibody levels due to prior infection. Additionally, there is a lag between infection and the production of antibodies (10–12 days). Thus, symptomatic individuals could have low Pox-specific antibody levels due to sampling prior to antibody production. These factors confounded our ability to detect relevant differences in Pox-specific antibody levels within a population.

In contrast, we were able to compare *Philornis*-specific antibody levels between unexposed and exposed birds from El Garrapatero, because we could determine the timing of parasite exposure (nesting period), visually confirm the presence of the parasite, and obtain blood samples after the lag time required for up-regulation of any antibody response. Although pre-nesting birds could have been exposed to *Philornis* in a previous breeding season, and thus have anti-*Philornis* antibodies, we expected those antibody levels to be low (at or near background), owing to the breakdown of antibodies in the absence of antigenic stimulation between breeding seasons [24].

Antigen Production

First and second instar larvae of *P. downsi* were used for antigen extraction. Larvae were placed into a centrifuge tube and macerated with 100 µL of phosphate buffered saline (PBS) and 1mM EDTA. The tube was centrifuged at 14.8 thousand revolutions per minute, and the supernatant containing the extract was removed. The supernatant was passed through a 0.2 micron filter and the protein concentration was estimated using a spectrophotometer. The extract was diluted to a concentration of 0.613 mg mL^{-1}.

For pox antigen we used a live virus vaccine for Fowl Pox Virus (FP-VAC; Intervet/Schering-Plough), following tests of binding by Darwin's finch antibodies (see below) and based on the likely occurrence of conserved antigens among Fowl Pox and Canary Pox [25].

Production of Secondary Antibody and Cross Reactivity with Darwin's Finch Serum

Anti-house-sparrow-immunoglobulin antiserum was produced by immunizing rats with purified house sparrow (*Passer domesticus*) IgY (Yolk Immunoglobulin).

House sparrow IgY was isolated using thiophilic interaction chromatography (described in 26). The recovered fraction was analyzed via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 12% slab-gels and stained with Coomassie Blue R-250 to confirm the presence of house sparrow IgY.

Lyophilized house sparrow IgY was then re-dissolved in PBS at 1 µg/µl and emulsified with an equal volume of complete Freund's adjuvant (CFA). Three rats received a subcutaneous primary injection of house sparrow IgY with CFA (50 µg of protein/100 µl emulsion was used per injection). Rats received booster shots containing house sparrow IgY with incomplete Freund's adjuvant (IFA) at 4-week intervals two times. Rats were exsanguinated 4 weeks after the final booster shot.

Cross-reactivity between house sparrow IgY, Darwin's finch serum and the rat antiserum was confirmed using Western-Blot analysis. Briefly, purified IgY was separated using SDS-PAGE and transferred on to a nitrocellulose membrane for immunoblotting. Filters were blocked with casein blocking buffer for one hour at room temperature and then washed three times in double deionized water (ddH2O). The blots were incubated for one hour at room temperature with rat-anti-house-sparrow-IgY (RαHOSP-IgY) and then washed three times again with ddH2O. The blots were then incubated for another hour at room temperature with commercially prepared goat-anti-mouse antibody conjugated to horseradish peroxidase (GαM-hrp) (Bethyl Laboratories, Inc., Mongomery, TX) and then washed a final three times with ddH2O. The blots were analyzed using enhanced chemiluminesence (Figure 2).

Cross-reactivity between Darwin's finch serum and RαHOSP-IgY was established via enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well ELISA plates were coated in triplicate with 100 µl of Darwin's finch serum diluted at 1:100, 1:500,

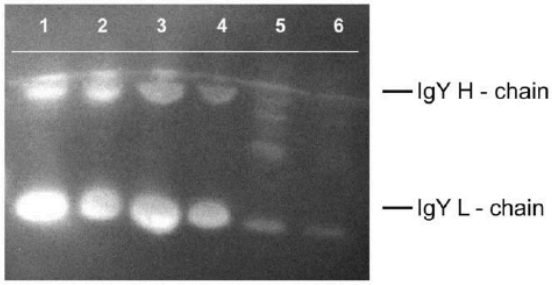

Figure 2. Western blot of serum dilutions developed for house sparrow IgY. Western blot of serum dilutions from Darwin's finch (DF), house sparrow and chicken using antibody markers developed for house sparrow IgY. Lane 1: DF serum 1:10. Lane 2: DF serum 1:20. Lane 3: house sparrow serum 1:10. Lane 4: house sparrow serum 1:20. Lane 5 chicken serum 1:10. Lane 6 chicken serum 1:20. Image indicates cross reactivity of house sparrow IgY detection antibody with Darwin's finch IgY. The lack of binding to chicken serum indicates no cross-reactivity with that species.
doi:10.1371/journal.pone.0008605.g002

1:1000, and 1:5000 in carbonate coating buffer (0.05 M, pH 9.6). The plates were incubated for one hour at 37°C on an orbital table before being washed three times with 200 µl of wash solution per well. The plates were blocked with casein blocking buffer and again incubated for one hour at 37°C on an orbital table. The RαHOSP-IgY was diluted in sample buffer at 1:50, 1:100, 1:500 and 1:1000. After washing the plate three times, 100 µl of the RαHOSP-IgY was added to each Darwin's finch serum dilution, such that each serum dilution was tested against each RαHOSP-IgY dilution. Plates were again incubated for one hour at 37°C on an orbital table and then washed three times. The secondary antibody, GαM-hrp, was diluted 1:1000 in sample buffer and 100 µl of this solution was added to each well. The plates were incubated for one hour at 37°C on an orbital table and then washed a final three times. 100 µl of peroxidase substrate (2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid, ABTS: Sigma cat. A1888) and peroxide was added to each well and the plates were covered with tinfoil and allowed to develop for one hour at room temperature before being read on a spectrophotometer using a 405-nanometer filter. Three blank wells were included on each plate, as well as three wells that measured non-specific binding, which quantified binding of RαHOSPIgY and GαM-hrp to the respective antigen. These wells received all the reagents described above except for Darwin's finch serum. In this step, blocking buffer was used in place of serum. The mean absorbance of these wells was subtracted from the absorbance measures determined above. Results from this ELISA indicated crossreactivity between Darwin's finch serum and RαHOSP-IgY.

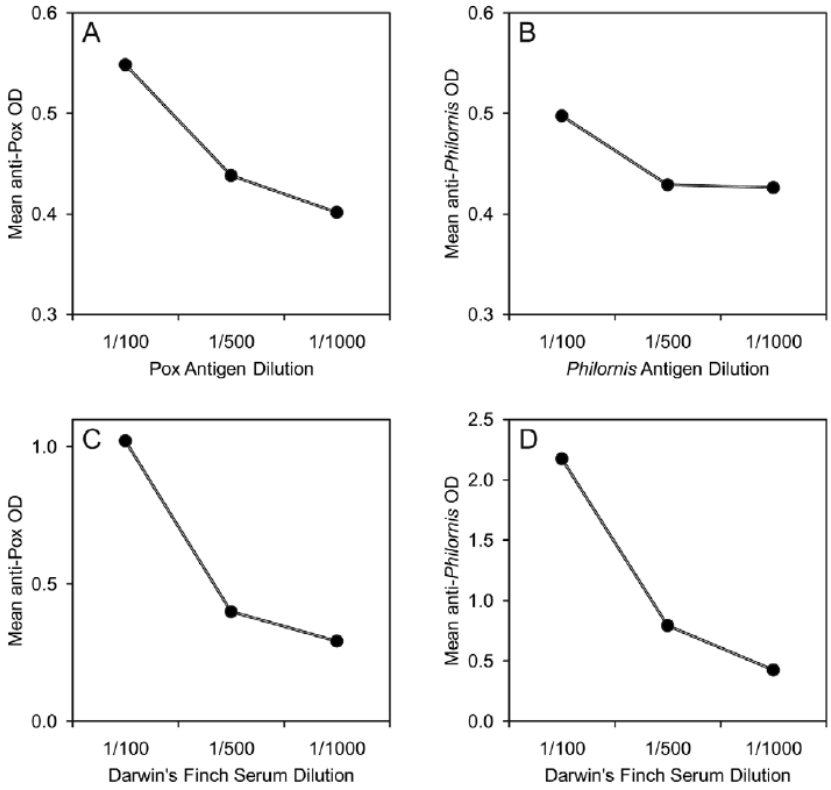

Figure 3. Optimization of ELISAs for antigen and Darwin's finch serum. Optical density (OD) values for optimization ELISAs of (A) Pox antigen dilutions and Darwin's finch serum at 1/500, (B) *Philornis* antigen dilutions and Darwin's finch serum at 1/500, (C) Darwin's finch serum dilutions and Pox antigen at 1/1000, and (D) Darwin's finch serum dilutions and *Philornis* antigen at 1/1000. Decreasing amounts of antigen (A,B) and antibody (C,D) result in decreasing optical density values, indicating specific antibody-antigen binding.
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Cross Reactivity of Darwin's Finch Antibiodies and Parasite Antigen

Cross-reactivity between Darwin's finch antibodies and *Philornis downsi* protein, or Fowl Pox virus, was established via ELISA, using dilutions of Darwin's finch serum and antigen (*Philornis* protein or Fowl Pox virus). Briefly, 96-well ELISA plates were coated in triplicate with 100 µl of either Fowl Pox virus in PBS, or *Philornis* extract, diluted at 1:100, 1:500, or 1:1000 in carbonate coating buffer (0.05 M, pH 9.6). Plates were incubated for one hour at room temperature on an orbital table, and then washed five times in wash buffer. 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 Darwin's finch serum (pooled sample) then diluted 1:100, 1:500 or 1:1000 in sample buffer, such that each serum dilution was tested against each antigen dilution. Plates were incubated for one hour at room temperature on an orbital table, and then washed (5×) with wash buffer. Next, 100 µl of RαHOSP-IgY (1:1000) was added to each well, followed by a one hour incubation at room temperature and wash (5×). The second detection antibody (GαM-hrp, 1:1000) was then added, followed by a one hour incubation at room temperature and washing (5×). Finally, 100 µl of peroxidase substrate (tetramethylbenzidine, TMB: Kirkegaard and Perry cat. 50-77-03) was added to each well. The plates were incubated for exactly five 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. Based on optimization results (Figure 3), a standard serum dilution of 1:500 was selected for the ELISAs of individual birds and a standard dilution of 1:1000 was selected for Pox and *Philornis* antigens, which were tested separately.

On each plate we included three wells for non-specific binding, which quantified binding of RαHOSP-IgY and GαM-hrp to the respective antigen. These wells received all the reagents described above except for Darwin's finch serum. In this step, blocking buffer was used in place of serum. The mean absorbance of these wells was subtracted from the absorbance measures determined above. Finally, we calibrated absorbance values between plates using a positive control. In brief, each plate contained the same reference sample in triplicate. The reference sample absorbance was compared across all plates, and we calculated a correction factor for each plate to standardize absorbance. These standardized values were used for subsequent analyses of immune response in birds.

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Author Contributions

Conceived and designed the experiments: SKH JPO DHC. Performed the experiments: SKH JAHK MOK PRG BRG DHC. Analyzed the data: SKH JPO JAHK MOK DHC. Contributed reagents/materials/analysis tools: SKH JPO JAHK MOK PRG BRG DHC. Wrote the paper: SKH JPO JAHK MOK PRG BRG DHC.

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CHAPTER 4

TEST FOR PARASITE-SPECIFIC IMMUNE RESPONSE IN MULTIPLE SPECIES OF DARWIN'S FINCHES

Abstract

Ecoimmunology aims to explain variation in immune responses within an ecological and evolutionary context. Traditionally, studies have used non-pathogenic agents to elicit nonspecific immune responses in hosts. Studies of immune responses to specific parasites are often limited to host species for which commercially produced detection antibodies are available. Recently, medium ground finches (*Geospiza fortis*) were shown to mount a parasite-specific antibody-mediated immune response to the introduced ectoparasite, *Philornis downsi*, using an indirect enzyme-linked immunosorbent assay (ELISA) with house sparrow (*Passer domesticus*) antiserum. Despite these two species of birds not being closely related, house sparrow antiserum cross-reacted well with medium ground finch serum. This study validates the use of house sparrow antiserum to quantify parasite-specific immune responses in other species of Darwin's finches. *P. downsi* was recently introduced to the Galapagos and is known to negatively affect nestling growth and fledging success in several species of finches. Validation of this immuno-assay with other species of Darwin's finches is the first step toward determining whether these species are also able to mount *P. downsi*-specific

antibody-mediated immune responses and in determining whether this immune response is a viable defense mechanism against *P. downsi*.

Introduction

The field of ecoimmunology explores variation in immune responses relative to tradeoffs with other life-history traits (Norris & Evans, 2000, Lochmiller & Deerenberg, 2000). Traditionally, studies have used derived substances (e.g., phytohaemagglutinin (PHA), keyhole limpet hemocyanin (KLH)) or attenuated pathogens (Newcastle disease virus vaccine (NDV)) to elicit various immune responses in host organisms (Hasselquist et al., 2001, Smits et al., 1999, Saino et al., 2002). This approach allows researchers to compare life-history traits between individuals while eliminating the confounding effects of a given parasite or pathogen on host fitness. In addition, the substances used to elicit these responses are commercially available and easy to use in a field setting (Martin et al., 2004). While this approach has provided useful insights about potential trade-offs between the immune system and other fitness components, it ignores the more complicated interactions that can occur between a host and parasite (Owen & Clayton, 2007, Kennedy & Nager, 2006, Norris & Evans, 2000, Owen et al., 2010). Immune responses to substrates, such as PHA or KLH, may not be comparable in longevity or intensity to immune responses elicited by actual parasites (Owen & Clayton, 2007). Thus, the field of ecoimmunology is evolving to assess the costs and benefits of host immune responses to relevant parasites and pathogens.

Unfortunately, quantifying host immune responses to real parasites and pathogens is still largely limited by the techniques and reagents available for a given host species. Most work on avian immunology is still centered on poultry species due to their

agricultural and economic influence. The majority of commercially available antibody and immuno-assay products are designed for domestic chickens (*Gallus gallus domesticus*). Low cross-reactivity between chicken antiserum and many wild bird species limits the number of systems in which avian antibody responses can be studied using chicken antiserum. However, recent efforts have been made to design other antibody products for wild bird species (King et al., in press, Ilmonen et al., 2002, Hasselquist et al., 1999). King et al. (in press) created antiserum against purified house sparrow (*Passer domesticus*) immunoglobulin Y (IgY) and tested its cross-reactivity with a variety of other passerine and non-passerine species. Using tests of dilutional parallelism, the authors showed that house sparrow antiserum cross-reacted strongly with eight of 19 wild bird species tested.

The development of house sparrow antiserum provided the opportunity to study antibody-mediated immune responses in rarer bird species, including Darwin's finches (Huber et al., 2010). For high-yield production of antiserum, destructive sampling of eggs is required to extract immunoglobulins from the yolk (De Meulenaer & Huyghebaert, 2001, King et al., in press). Since destructive sampling of large numbers of Darwin's finch eggs is undesirable, it is not feasible to develop antiserum specific to this group of birds. However, Huber et al. (2010) successfully quantified parasite-specific antibody-mediated immune responses (IgY) in populations of medium ground finches (*Geospiza fortis*) using house sparrow antiserum in enzyme-linked immunosorbent assays (ELISA). Their results showed that medium ground finches mount antibody-mediated immune responses specific to the recently introduced nest parasite, *Philornis downsi*.

Philornis downsi (Diptera: Muscidae) is a parasitic fly that was recently introduced to the Galapagos Islands, and which has the potential to affect many bird species, including all species of Darwin's finches (Fessl & Tebbich, 2002). Adult flies lay their eggs in the nests of birds. The eggs hatch and larvae blood-feed on nestling and adult birds as they progress through three instars. The larvae then pupate in the nest material and emerge as adult flies. Adult flies are nonparasitic and feed on organic matter (Dodge & Aitken, 1968, Couri, 1985). *P. downsi* has been documented on 11 of 13 major islands in the archipelago and in the nests of at least 14 species of birds, including 9 species of Darwin's finches (Wiedenfeld et al., 2007, Fessl & Tebbich, 2002). *P. downsi* significantly reduces nestling growth and fledging success in several finch species (Koop et al., in press, Fessl et al., 2006). In fact, *P. downsi* has been implicated in the recent severe declines of the critically endangered medium tree finch (O'Connor et al., 2009), mangrove finch (Fessl et al., 2010) and warbler finch (Grant et al., 2005).

The goal of this study was to validate the use of house sparrow antiserum to detect antibody-mediated immune responses in several species of Darwin's finches. The high relatedness between species of Darwin's finches (Grant, 1986) suggests that house sparrow antiserum should cross-react similarly between species of Darwin's finches. Since *P. downsi* is known to parasitize multiple species of Darwin's finches, we would predict that these species will also mount *P. downsi*-specific antibody-mediated immune responses.

Methods

Study site

Our study was conducted January-April, 2009 on Santa Cruz Island in the Galapagos Archipelago, Ecuador. Santa Cruz Island has three main geographic areas, including an arid zone around the perimeter of the island, a humid, highland zone at the central peaks, and a transitional/agricultural zone between these. Samples were collected from 8 species of adult Darwin's finches where populations are most abundant. Samples from adult *Geospiza fortis, G. magnirostris, G. scandens*, and *Platyspiza crassirostris* were collected only in the arid zone. *Certhidea olivacea* and *Camarhynchus parvulus* were collected only in the highland zone. *Cactospiza pallida* and *Geospiza fuliginosa* were collected in both the highland and arid zones.

We used mist nests to capture adult birds in each habitat. Upon capture, we collected a small blood sample (70 μl) via brachial veinipuncture. Blood was collected using a heparinized hematocrit tube and transferred to a 1.5 ml microcentrifuge tube for storage in a cooler of wet ice. Bleeding stopped within 1 minute of pressure being applied at the puncture sight. Birds were immediately released following processing. Within six hours of collection, each blood sample was spun by hand-crank centrifuge for 5 minutes. Plasma was extracted from each vial and transferred to a separate 1.5 ml microcentrifuge vial for storage. All vials were then placed in a -20°C freezer until the end of the field season. Upon return to the United States, blood samples were stored in a -80°C freezer until further processing.

Huber (2010) found that female medium ground finches had greater *P. downsi*-specific immune responses than males. Therefore, we used plasma from females in our

study to maximize the likelihood of detecting *P. downsi*-specific antibodies. Furthermore, we used pooled samples of plasma from six individual females of each species of Darwin's finch for the assays.

Cross-reactivity validation with αHOSP-IgY

To validate that house sparrow antiserum (αHOSP-IgY) cross-reacted with plasma from various Darwin's finch species, we performed a sandwich ELISA for total IgY and a test of dilutional parallelism for each species (Plikaytis et al., 1994, Washburn et al., 2007). Pooled plasma from each species was used to make the following serial dilutions in sample buffer (Tris-buffered saline with 0.05% Tween 20): 1:1000, 1:2000, 1:5000, 1:10,000, 1:15,000, 1:20,000, 1:25,000, 1:50,000. All samples were run in triplicate.

The following protocol is modified from King et al. (in press). Briefly, 96-well plates were coated with 100 µl/well of Rat-αHOSP-IgY plasma diluted 1:1000 in coating buffer (sodium bi-carbonate, 0.05M, pH 9.6) and incubated overnight at 4°C. Plates were loaded with 200µl/well of blocking buffer (Tris-buffered saline with bovine serum albumin, pH 8.0) and incubated at 37°C for 30 minutes on an orbital table. Between each of the following steps, plates were washed five times with wash buffer (Tris-buffered saline with Tween 20, pH 8.0), loaded as described below and incubated at 37°C on an orbital table for 1 hour. Plates were loaded with 100 µl/well with the eight finch species plasma dilutions (three wells per species per dilution). Plates were then loaded with 100 µl/well of Rabbit-αHOSP-IgY diluted 1:1000 in sample buffer. Plates were then loaded with 100 µl/well of Goat-αRabbit-hrp (conjugated detection antibody; Bethyl Laboratories, A120-101P) diluted 1:20,000 in sample buffer and covered with aluminum

foil for the incubation step. Following a final wash, plates were loaded with 100 μl/well of TMB (tetramethylbenzidine, TMB: KPL 50-76-00) and incubated at room temperature for exactly ten minutes. Immediately following this step, the reaction was stopped using 100 μl of 2 M H_2SO_4 in each well. Optical density (OD) was measured using a spectrophotometer (BioTek, PowerWave HT, 450-nanometer filter).

On each plate, a positive control of pooled *G. fortis* plasma (diluted 1:500) was used in triplicate to control for interplate variation. In addition, each plate was also run with a nonspecific binding (NSB) sample in which Rat-αHOSP-IgY, Rabbit-αHOSP-IgY, and Goat-αRabbit-hrp were added but not plasma, and a blank sample in which only Rabbit-αHOSP-IgY and Goat-αRabbit-hrp were added but not plasma or Rat-αHOSP-IgY. NSB absorbance values were subtracted from each sample value on a given plate. In addition to the eight Darwin's finch species tested, a negative control of pooled chicken plasma was also run at each sample dilution.

Intraassay and interassay variation was calculated as the %CV ((SD/grand mean) *100)). If the %CV was > 10-15%, the sample or plate in question was rerun. Mean OD values for each species were log-transformed and plotted as a function of their dilution. A linear regression line was calculated for each species and the slopes of those lines were compared using a one-way ANOVA. We used Dunnett's multiple comparison post-hoc tests to compare the slope of each line to that of *G. fortis*. If the slope of the line for a given species was not significantly different from the slope for *G. fortis*, that species was considered to have equal cross-reactivity with house sparrow antiserum.

Test for *P. downsi*-specific antibody response

To test whether each species produced *P. downsi*-specific antibodies, we used reciprocal indirect ELISAs to test responses to increasing dilutions of *P. downsi* antigen and increasing dilutions of host plasma. The protocol was modified slightly from that described in Huber et al. (2010).

For the antigen dilution assay, 96-well ELISA plates were coated with 100 µl/well of *P. downsi* antigen diluted (stock concentration: 26 µg/ml) 1:1000, 1:2000, 1:5000, 1:10,000, 1:15,000, 1:20,000 in coating buffer. Plates were covered and incubated overnight at 4°C. Following overnight incubation, plates were coated with 200 µl/well of blocking buffer. Between each of the following steps, plates were washed five times with wash buffer (Tris-buffered saline with Tween 20, pH 8.0), loaded as described and incubated at room temperature on an orbital table for 1 hour. Plates were loaded with 100 µl/well of pooled plasma from each species diluted 1:500 in sample buffer. Plates were loaded with 100 µl/well of Rabbit-αHOSP-IgY diluted 1:10,000 (secondary antibody made by MOK). Plates were then loaded with 100 µl/well of detection antibody Goat-αRabbit-hrp (1:20,000) and covered with aluminum foil. Finally, plates were loaded with 100 µl/well of TMB peroxidase substrate and incubated for exactly ten minutes at room temperature. Immediately following this incubation step, the reaction was stopped using 100 µl of 2 M H_2SO_4 in each well. Optical density (OD) was measured using a spectrophotometer (450-nanometer filter).

For the plasma dilution assay, plates were coated with *P. downsi* antigen diluted at 1:1000 in coating buffer. Pooled plasma samples were diluted in sample buffer at the

following concentrations: 1:100, 1:500, 1:1000, 1:2000, 1:5000, 1:10,000. All other steps and procedures were identical to the antigen dilution assay described above.

All samples were run in triplicate. Each plate had an NSB sample, a blank sample and a positive control sample as a reference for inter-plate variation. The NSB value was subtracted from the OD value for each sample.

Results

Cross-reactivity validation with αHOSP-IgY

Overall, slopes generated from linear regression analysis differed significantly between species ($F_{8,63} = 31.33$, $p < 0.0001$). Species that cross-reacted with αHOSP-IgY similarly to *G. fortis* had species-specific slopes that were not significantly different from the slope generated for *G. fortis* plasma (Table 4.1; Fig. 4.1). This was the case for every species of Darwin's finch except *G. scandens* (Dunnett's post-hoc, $p > 0.05$). The slope for all species of Darwin's finches significantly differed from the slope generated from chicken serum, which served as a negative control (Dunnett's post-hoc, $p < 0.05$).

Test for *P. downsi*-specific antibody response

All eight species of finches tested had *P. downsi*-specific antibodies present. Increasing dilutions of *P. downsi* antigen resulted in decreasing optical density values in all species of Darwin's finches tested (Fig. 4.2A). In the reciprocal assay, increasing dilutions of plasma resulted in decreasing optical density values in all species of Darwin's finches tested (Fig. 4.2B).

Table 4.1. Results of Dunnett's multiple comparison post-hoc test of slopes between *Geospiza fortis* and other species of Darwin's finches relative to their cross-reactivity with house sparrow antiserum. Chicken (*Gallus gallus domesticus*) plasma was used as a negative control.

Species (label)	**Slope ± SE**	**Slope significantly different from *G. fortis*? (p > 0.05)**
Geospiza fortis	310.2 ± 31.71	n/a
Platyspiza crassirostris	332.0 ± 37.44	No
Geospiza fuliginosa	316.6 ± 32.96	No
Geospiza magnirostris	307.4 ± 35.01	No
Certhidea olivacea	317.2 ± 48.53	No
Cactospiza pallida	287.7 ± 35.04	No
Camarhynchus parvulus	332.6 ± 44.28	No
Geospiza scandens	250.6 ± 32.86	Yes
Gallus gallus domesticus	109.1 ± 9.254	Yes

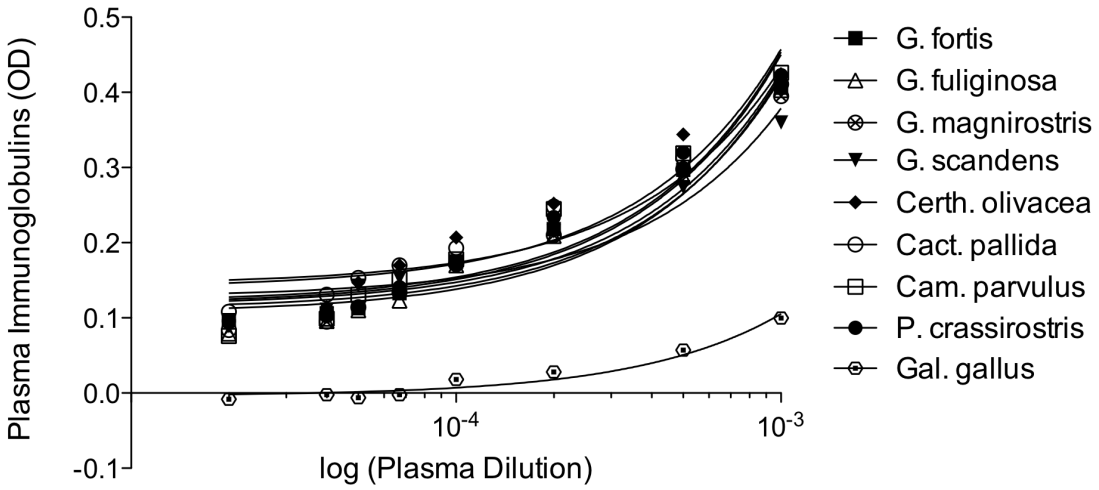
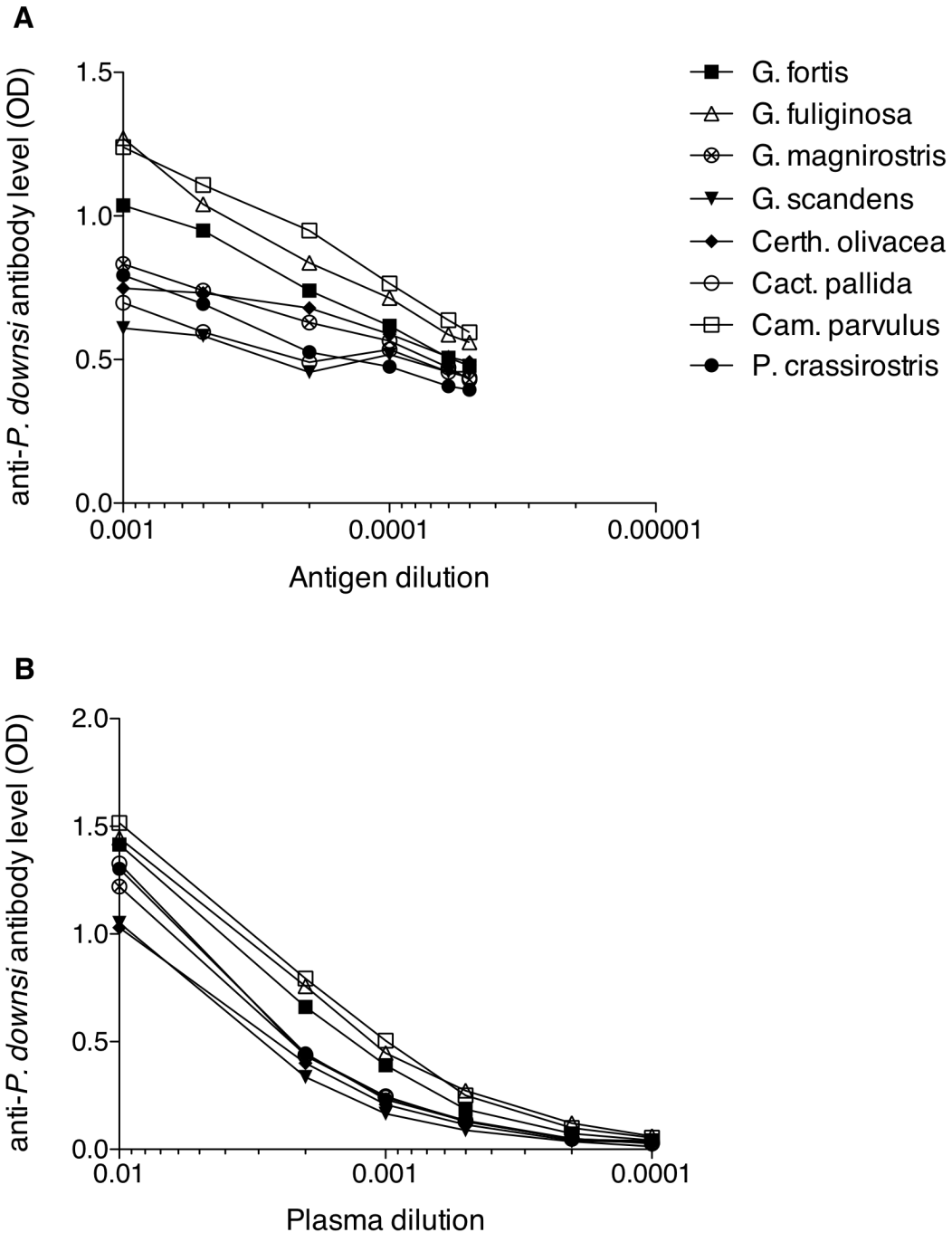


Figure 4.1. Comparison of cross-reactivity of house sparrow antiserum with plasma from different Darwin's finch species. Optical density (OD) values from the total IgY enzyme linked immunosorbent assay (ELISA) are shown as a function of decreasing plasma concentrations for eight species of Darwin's finches. Chicken (*Gallus gallus domesticus*) plasma was used as a negative control.

Figure 4.2. Results of enzyme-linked immunosorbent assays (ELISA) to test whether different species of Darwin's finches mount *P. downsi*-specific antibody responses. (A) The optical density (OD) values for Darwin's finch species are shown relative to decreasing *P. downsi* antigen concentrations when finch plasma was run at a constant dilution (1:500). (B) The optical density (OD) values for Darwin's finch species are shown relative to decreasing plasma concentrations when *P. downsi* antigen was run at a constant dilution (1:1000). Decreasing OD values in response to decreasing antigen or serum concentrations indicate specific antibody-antigen binding.


A
anti-P. downsi antibody level (OD)
1.5
1.0
0.5
0.0
0.001
0.0001
0.00001
Antigen dilution
G. fortis
G. fuliginosa
G. magnirostris
G. scandens
Certh. olivacea
Cact. pallida
Cam. parvulus
P. crassirostris
B
anti-P. downsi antibody level (OD)
2.0
1.5
1.0
0.5
0.0
0.01
0.001
0.0001
Plasma dilution

Discussion

HOSP-IgY antiserum can be used to detect antibody responses in at least eight species of Darwin's finches. Six of the seven Darwin's finch species cross-reacted with house sparrow antiserum similarly to *G. fortis*. As expected, domestic chicken serum served as a negative control and did not cross-react with HOSP-IgY antiserum (King et al., in press, Huber et al., 2010). The slope for *G. scandens* also significantly differed from *G. fortis* (Table 4.1) *G. scandens* OD values differed from the other Darwin's finch species only at very high plasma concentrations. More importantly, the slope of the line generated for *G. scandens* cross-reactivity differed significantly from the negative control, chicken. Thus, *G. scandens* is still considered to have relatively high cross-reactivity with HOSP-IgY antiserum. Further research is needed to determine whether the lower OD values for *G. scandens* in the total IgY assay indicate lower-antibody binding affinity with anti-HOSP IgY or lower antibody titers in this species.

Our study also demonstrates that eight species of Darwin's finches produce *P. downsi*-specific antibodies. In all species of Darwin's finches tested, OD values decreased with increasing plasma or *P. downsi*-antigen dilutions. This reciprocal relationship is indicative of specific antibody-antigen binding. The presence of parasite specific antibodies in eight different species of Darwin's finches may indicate that these host species all have a common line of defense against *P. downsi*.

Antibody-mediated immune responses can be an effective defense against ectoparasites (Wikel, 1996). The production of parasite specific antibodies can increase the speed and intensity with which inflammation can occur at the site of a bite (Owen et al., 2010). Inflammation, or thickening of the skin, prevents blood-feeding parasites from

being able to easily access capillary beds. Inflammation can decrease the feeding ability of parasites, leading to parasite mortality (Owen et al., 2009). Further study is needed to determine whether a *P. downsi*-specific immune response by Darwin's finches is an effective defense mechanism against this parasite.

In some species, female birds can transfer maternally produced antibodies to their offspring (Boulinier & Staszewski, 2008). Nestling finches are likely the primary food source for *P. downsi* larvae since they are relatively immobile and defenseless in the nest, compared to adults. Nestlings that obtain maternally transferred antibodies would therefore benefit. Maternally transferred antibodies can have a very short half-life in some passerine nestlings, lasting only 2-4 days (King et al., 2010). Therefore, nestlings would presumably also need to endogenously produce antibodies to begin or continue defending themselves against *P. downsi*. Further work is needed to determine whether maternal transferred antibodies occur in any of these finch species, and whether nestling finches are able to produce effective antibody responses to *P. downsi*.

Our study shows that house sparrow antiserum cross-reacts well with at least eight species of Darwin's finches, a group of birds for which de novo development of host-specific antiserum is not practical. The development of reagents that cross-react well with wild bird species expands the number of systems in which studies of ecoimmunology can be pursued.

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CHAPTER 5

ARE DARWIN'S FINCHES SITTING DUCKS? INEFFECTIVE HOST DEFENSES AGASINT AN INTRODUCED PARASITE

Abstract

Hosts use a variety of means to defend themselves against the harmful effects of parasites. However, hosts affected by introduced parasites may be unable to rapidly evolve effective defense mechanisms. The recent introduction of *Philornis downsi* to the Galapagos Islands poses a major threat to endemic bird species across the archipelago, including Darwin's finches. *P. downsi* is a hematophagous nest parasite known to significantly reduce host nestling growth and fledging success. The goal of our study was to investigate whether the medium ground finch (*Geospiza fortis*), a species of Darwin's finch, possesses defense mechanisms against *P. downsi* that are effective in negating at least some of the negative effects of this parasite. We performed an experimental manipulation using a fumigant to reduce *P. downsi* abundance in nests and monitored nestling growth and fledging success in fumigated and control nests. Nest cameras recorded parental and nestling behaviors during the day and at night in order to compare possible behavioral defenses in fumigated versus control nests. We used an enzyme linked immunosorbent assay (ELISA) to quantify *P. downsi*-specific

antibody responses in parent and nestling finches in fumigated and control nests. We found that adult female finches produce *P. downsi*-specific antibodies that may reduce parasite abundance in nests. Nestlings were not capable of producing *P. downsi*-specific antibodies, nor were maternally transferred antibodies present when nestlings were five days old. We found no evidence of effective behavioral defenses by parent or nestling finches, though observed alterations in behavior revealed possible mechanisms by which *P. downsi* cause nestling mortality. We observed significant negative effects of *P. downsi* on nestling growth. In addition, we observed extremely high levels of mortality in control nests (100% mortality in all 22 sham-fumigated nests). By comparison, all fumigated nests (21 nests) successfully fledged at least one offspring. In summary, our results suggest that medium ground finches do not possess effective behavioral or immunological defense mechanisms against *P. downsi*.

Introduction

Parasites are costly to their hosts. To reduce these costs, host have evolved a variety of defense mechanisms to control or eliminate their parasites (Clayton et al., 2010, Sheldon & Verhulst, 1996, Hart, 1990). In turn, parasites evolve means of escaping host defenses. Thus, hosts and parasites can coevolve through an arms race where populations fluctuate, but remain relatively stable over time. Parasites introduced to naïve hosts pose a particular threat if the hosts they infest lack or are unable to rapidly evolve effective defense mechanisms. This imbalance of arms between introduced parasites and naïve hosts can lead to rapid population declines in one or both groups of organisms.

Introduced parasites are of growing global concern for both human and wildlife populations (Cleaveland et al., 2002, Daszak et al., 2000). Small populations of hosts, like those on islands, are particularly vulnerable to the effects of introduced parasites. For example, the introduction of avian malaria and its mosquito vector to the Hawaiian Islands by humans has been implicated in the severe decline or extinction of several endemic bird species (van Riper et al., 1986, Warner, 1968). Decades after the introduction, the presence of these parasites has selected for populations of birds that have evolved immunogenetic and behavioral defenses (van Riper et al., 1986). An initial rapid decline in host populations suggests that hosts were naïve to avian malaria and did not have effective defenses in place when this parasite was first introduced.

No endemic species of birds have gone extinct over recorded history in the Galapagos Islands, probably because of their late colonization by humans in the late 1800's (Bensted-Smith 2002). However, recent estimates show that the human population on the islands is increasing annually by 4% (Watkins & Cruz, 2007). Expanding cities and agricultural zones have led to dramatic increases in the number of parasites introduced to the islands (Wikelski et al., 2004). A parasite of particular concern for Galapagos bird species is the parasitic fly *Philornis downsi* (Diptera: Muscidae). Larvae of *P. downsi* were first observed in the nests of birds in 1997 (Fessl & Tebbich, 2002). *P. downsi* has been found on 11 of the 13 major islands (Wiedenfeld et al., 2007) and has been documented in the nests of 14 species of birds on the Galapagos, including 9 species of Darwin's finches (Fessl & Tebbich, 2002, O'Connor et al., 2009, Grant et al., 2005).

P. downsi is an obligate nest ectoparasite of birds (Couri, 1985, Dodge, 1971). Adults lay their eggs in the nests of birds where the larvae hatch and progress through three instars. Larvae crawl up from the bottom of the nest and blood-feed on nestling, and possibly adult birds, by chewing through the skin and consuming the secreted fluids. Third instar larvae pupate in the nest material and later emerge as adult flies (Fessl et al., 2006). Adult flies are non-parasitic and feed on organic matter. Nestlings are the primary food source for feeding larvae because they are immobile in the nest.

Previous studies have documented the negative effects of *P. downsi* on nestling growth and fledging success (reviewed in Koop et al., 2011). However, few studies have examined potential host defense mechanisms against *P. downsi*. In one of the first demonstrations of a parasite-specific antibody response in a wild population of birds, Huber et al. (2010) showed that adult medium ground finches (*Geospiza fortis*) mount *P. downsi*-specific antibody responses. In other systems, specific antibody responses have been shown to increase the speed at which inflammation occurs (Owen et al., 2009). Inflammation, which leads to a thickening of the skin, can prevent blood-feeding parasites from accessing host capillary beds. Ectoparasites that feed on inflamed host tissues can also imbibe proteolytic enzymes that directly damage parasite tissues (Owen et al., 2010). Huber et al. (2010) showed that breeding adult finches have greater *P. downsi*-specific antibody responses than pre-breeding adults. Furthermore, females have greater responses than males, consistent with the hypothesis that females have increased exposure to *P. downsi* while they brood nestlings (males do not brood).

In altricial birds, nestlings are born without fully developed immune systems (Apanius, 1998). However, King et al. (2010) showed that nestling house sparrows

(*Passer domesticus*) can produce antibodies endogenously within 3-6 days after hatching. To date, no studies have investigated whether nestling Darwin's finches are capable of producing *P. downsi*-specific antibodies. Nestlings able to produce antibodies against *P. downsi* may be able to negate some of the negative effects of parasitism. Immune responses by adult females may also benefit nestlings if mothers are able to transfer antibodies to their offspring. Females of some bird species can transfer circulating antibodies prenatally to their eggs following exposure to an antigenic challenge (Boulinier & Staszewski, 2008). Maternally transferred antibodies could act similarly to nestling endogenously produced antibodies in negating the effects of parasitism.

Parents may be able to further negate the effects of parasitism on their nestlings by altering their behavior. Parents can increase food provisioning to nestlings to offset some of the energetic costs imposed by parasites (Hurtrez-Bousses et al., 1998). Parents may also increase nest sanitation behavior to control the number of parasites in the nest (Christe et al., 1996b). For example, O'Connor (2010) observed Darwin's finch parental behavior in nests of small (*Geospiza fuliginosa*) and medium ground finches parasitized by *P. downsi*. Parents were observed probing the nest material and allopreening nestling feathers and nares presumably to remove *P. downsi* larvae. Nestlings in heavily parasitized nests were observed preening and repositioning themselves, though no clear relationship was found between nestling or parental behaviors and fledging success.

We experimentally manipulated *P. downsi* abundance in the nests of medium ground finches to investigate the role of immunological and behavioral defenses in this system. We collected blood samples from breeding adults and nestlings from fumigated

and control nests to compare *P. downsi*-specific antibody responses relative to parasite abundance. We used nest cameras to record parental behavior in fumigated and control nests to identify and quantify possible defensive behaviors. Finally, we monitored nestling growth and fledging success in fumigated and control nests to evaluate the efficacy of these defenses against *P. downsi*.

Methods

Study site and experimental design

Our study was conducted January-April, 2009 and 2010, on the island of Santa Cruz, in the Galapagos Archipelago. Our field site, El Garrapatero, is a 1.5 x 1.5 km area in the arid, coastal zone. 2009 was a very dry year, resulting in a limited number of breeding medium ground finches at our field site (n = 13 total nests). The 2010 breeding season was much wetter, which supported a larger number of breeding finches at the same field site (n = 43 total nests). 2009 was used as a preliminary test of methods to ensure that fumigant could be used to control *P. downsi* abundance in nests. We report the effects of *P. downsi* parasitism on fledging in 2009 and 2010; however, all work on immunological and behavioral defenses is based on data collected only in 2010.

Medium ground finches are abundant at El Garrapatero where they nest primarily in giant prickly pear cacti (*Opuntia galapageia*) (Huber, 2008). Males and females both participate in nest building, but only females incubate eggs and brood hatched offspring. Both parents feed nestlings. Clutch size typically ranges from 2-5 eggs. Females incubate the eggs for 10-14 days before hatching occurs. Medium ground finch nestlings hatch asynchronously over a 2-4 day period. Nestlings then spend another 10-14 days in the nest prior to fledging. In years of adequate food resources, medium ground finches

lay multiple clutches within a given breeding season. Finches do not reuse nests between reproductive bouts or breeding seasons (Grant, 1986).

We searched El Garrapatero for medium ground finch nests by monitoring adult activity to identify mating pairs that had begun building a nest. Nests were checked every other day to determine the start date of egg laying and hatching. Nests were alternately assigned to the fumigant (n = 7 nests in 2009, 21 nests in 2010) or control (n = 6 nests in 2009, 22 nests in 2010) treatment. In all cases of re-nesting by a single pair (0% in 2009, 19% in 2010), the treatment was reversed between reproductive bouts. Nests were treated on the day of the first nestling hatching and again four days later. Nestlings and eggs were removed, along with a thin layer of nesting material, from the inner bottom of the nest. Fumigated nests were sprayed with a 1% pyrethrin solution (Permectrin II©) (Fessl et al. 2006) to eliminate parasites. Control nests were sham-fumigated with water. The nests were given several minutes to dry and then nestlings, eggs, and nesting material were returned to the nest. Parents were very quick to return to nests following treatment, and no cases of nest abandonment due to treatment were observed.

Active nests were visited every other day between the hours of 0600 and 1100 and the number of eggs and nestlings recorded. We continued to check nests until the oldest nestling was 10 days old, or until all of the nestlings died. Checking nests with nestlings older than 10 days can trigger premature fledging (Grant, 1981). Therefore, once the oldest nestling reached 10 days of age, we stopped checking the nests until the youngest nestling was observed to have fledged or the nest was empty. Nests of medium ground finches have a side entrance that makes it possible to determine nestling

presence from a distance with binoculars. Once empty, nests were collected to count parasites.

Parasite abundance

After each nesting bout we removed the nest and placed it in a sealed plastic bag. The nest was carefully dissected within 8 hours of collection and *P. downsi* larvae, pupae, and eclosed pupal cases were counted. First instar larvae, which are too small to discern reliably in the nest material, were not included in counts of parasite abundance. Total parasite abundance was therefore the sum of second and third instar larvae, pupae, and eclosed pupal cases.

Nestling growth

At each nest check the nestlings were weighed with a digital scale (Ohaus, 0.1g accuracy). In addition, the following measurements were taken with digital calipers (Fisherbrand, 0.01mm accuracy): bill length, bill depth, bill width, tarsus length, and length of the outermost primary feather, from where it emerged from the skin to its distal tip. At the first visit after hatching, nestlings were aged based on body mass using data from Boag (1984), as follows: ≤ 1.9 grams (1 day old); 2 - 2.9 grams (2 days old); 3 - 3.9 grams (3 days old). New nestlings were marked individually by coloring a toenail with a permanent marker. When nestlings were at least 7 days of age they were fitted with a numbered Monel metal band and three plastic color bands.

Fledging success

Successful fledging was confirmed by observing and identifying birds after they left the nest on the basis of their color band combinations. We also report on the number

of nestlings that survived to at least 9 days of age, but were not necessarily sighted after this time (see Kleindorfer et al., 2009).

Blood-sampling and hematocrit

Parent birds were captured using mist nests placed near the nest when the oldest nestling was approximately 5 days old. Adults were netted between the hours of 0600 and 0800. We measured adult tarsus length, mass, and wing chord. We collected a small blood sample (120 μl) by brachial venipuncture using a 27-gauge needle and heparinized capillary tubes. Cotton was applied with pressure immediately after collection until the blood clotted (< 1 minute). Birds were immediately released following banding, measurements and blood collection (< 15 minutes). Blood was stored on ice until further processing.

We also collected a blood sample (30 μl) from nestlings when they were approximately 5 days old using the same methods as for adults. Blood samples were taken during a normal nest check period to avoid additional disturbance at the nest. Cotton was applied with pressure immediately after collection and nestlings ceased to bleed within 30 seconds. Nestlings were placed back in the nest immediately following sampling.

Within 6 hours of collection, the blood was processed, and then moved to a -20°C freezer until the end of the field season. Capillary tubes were spun at 8000 rpm for 10 minutes in an automatic capillary centrifuge. Hematocrit was quantified by measuring the proportion of packed red blood cells relative to total blood volume. After hematocrit was measured, blood and plasma were transferred and stored in separate 1.5 ml microcentrifuge vials. Upon return to the United States, blood samples were kept in a

-80°C freezer until being processed in immuno-assays.

Immune response

We used enzyme-linked immunosorbent assays (ELISA) to detect the presence of *P. downsi*-specific antibodies in the plasma of Darwin's finches. The following protocol was modified slightly from that of Huber et al. (2010). Briefly, 96-well plates were coated with 100 µl/well of *P. downsi* extract (capture antigen) diluted in carbonate coating buffer (0.05M, pH 9.6). Plates were incubated overnight at 4°C, then washed and coated with 200 µl/well of bovine serum albumin (BSA) blocking buffer, and incubated for 30 minutes at room temperature on an orbital table. Between each of the following steps, plates were washed five times with a Tris-buffered saline wash solution, loaded as described below, and incubated for 1 hour on an orbital table at room temperature. Triplicate wells were loaded with 100 µl/well of individual finch plasma. Plates were then loaded with 100 µl/well of Rabbit-αHOSP-IgY (detection antibody; diluted 1:10,000) followed by 100 µl/well of Goat-αRabbit-hrp (secondary detection antibody; diluted 1:20,000) (Bethyl Laboratories). Finally, plates were loaded with 100 µl/well of peroxidase substrate (tetramethylbenzidine, TMB: Kirkegaard and Perry cat. 50-77-03) and incubated for exactly 10 minutes. The reaction was stopped using 100 µl/well of 2 M H_2SO_4. Optical density (OD) was measured using a spectrophotometer (BioTek, PowerWave HT, 450-nanometer filter).

For each plate a positive control of pooled plasma was used in triplicate to correct for interplate variation. In addition, each plate was also run with a nonspecific binding (NSB) sample in which capture antigen, detection antibody and secondary detection antibody were added, but not plasma, and a blank sample in which only the detection

antibodies were added, but neither plasma nor capture antigen. NSB absorbance values were subtracted from the mean OD value of each sample.

Behavior

We monitored nestling and parental activities using six battery-powered Sony® video camera systems. We placed small nest cameras (31 mm in diameter, 36 mm in length) equipped with infrared light sources in the top of nests. Medium ground finches make dome-shaped nests out of twigs and stiff grasses, which allowed the camera to be placed through a small hole made in the top of the nest without compromising structural integrity. Each camera had a 25 ft. cord that ran from the nest along the trunk of the cactus to the ground, and then approximately 10 feet away from the base of the cactus. The cord was attached to a small recording device (PV700 Hi-res DVR, 8 x 12 x 3 cm, stuntcams.com) that was hidden under brush.

Daytime behavior was recorded for approximately three hours between 0600 and 1000 in fumigated (n = 9 nests) and control (n = 9 nests) nests. Nighttime behavior was recorded for approximately two hours between 0100 and 0330 in fumigated (n = 5 nests) and control (n = 5 nests) nests. The infrared lights for nighttime video, which appear as very dim red lights from within the nest, do not affect nest success or predation (King et al., 2001, Staller et al., 2005). During the day, cameras were manually switched on and could be checked immediately for any malfunctions. At night, the cameras ran on timers in order to begin recording at 0100. Camera malfunctions at night resulted in a smaller sample size.

From the video recordings, we quantified the amount of time adults spent performing the following behaviors: feeding nestlings, nest attendance, nest sanitation,

brooding nestlings and allopreening nestlings. Females provide the majority of parental care and only females attend to the nest at night. Therefore, to make comparisons between daytime and nighttime parental behaviors, we report only the amount of time females spend attending to nests, and the subsequent behaviors performed while at the nest (ie. nest sanitation, brooding, allopreening). Nest sanitation was proportion of time the female actively searched the nest material with her bill out of the total time she was in attendance at the nest (Christe et al., 1996b). Brooding involved a female sitting directly on nestlings and was calculated as a proportion of time in attendance at the nest. Finally, allopreening was the proportion of time in which the female used her bill to preen any part of a nestling's body out of the total time that she was in attendance at the nest. Since both parents participate in feeding nestlings, and this behavior occurred only during the day, we report the total time both parents spent feeding nestlings as a proportion of the total time recorded.

We also quantified two nestling behaviors: preening and agitation. Preening was the proportion of time a nestling was moving its bill in contact with its plumage. Periods of agitation included shaking, repositioning or jumping in the nest. Parents at the nest often obscured the camera and decreased our ability to reliably see nestlings; therefore, we only quantified nestling behavior when parents were not present at the nest. Thus, nestling behaviors are reported as a proportion of the time nestlings were alone in the nest. Females were present throughout the night; therefore, we did not make observations of nestling behavior at night.

Due to a limited number of cameras and rapid nestling mortality, especially in sham-fumigated nests, it was not possible to collect video from each nest when nestlings

were of a consistent age. Therefore, for analyses, a single day/night of video from a control nest was matched to a single day/night of video from a fumigated nest based on nestling age and clutch size (n = 9 pairs of nests with daytime video, 5 pairs of nests with nighttime video). For example, behaviors were compared between a fumigated nest with three nestlings that were ~ 4 days old was matched to a sham-fumigated nest with three nestlings that were ~ 4 days old. Nestlings ranged in age from 2-6 days old and clutch size ranged from 1-5 nestlings. All videos were watched and scored by a single observer who was blind to nest treatment. Videos were analyzed using VLC media player (VideoLAN) and Quicktime 10.0 (Apple, Inc.).

Statistical analyses

All statistical analyses were performed in Prism® v.5.0b (GraphPad Software, Inc.) and R v.2.12.2 (R Development Core Team). All mean values are reported as the mean ± 1 standard error. Hematocrit values of adults and nestlings were compared between treatments using Mann-Whitney U tests.

We used linear mixed-effects models (R library "lme4" and the function "lmer") to compare the effects of treatment and age on growth parameters of nestlings in each treatment. Individual nestlings were defined as a random effect and clustered by nest since most nests had multiple nestlings. Age and treatment were fixed effects used to predict mass, tarsus length and outermost primary feather length. The model predicted growth (e.g., Y = mass) according to the equation ($Y = b_0 + b_1$*Treatment + b_2*Age + b_3*Treatment*Age). For control nests (Treatment = 0) the model predicting change in mass was reduced to $Y = b_0 + b_2$*Age, with b_0 as the intercept and b_2 as the slope of the line. For fumigated nests (Treatment = 1) the model predicting change in mass was

reduced to $Y = (b_0 + b_1) + (b_2 + b_3)*Age$. Thus, the coefficients b_1 and b_3 reflect the treatment effect on the slope and intercept of the line for nestlings in fumigated nests. We used AIC model selection ("ANOVA" in R) to assess the fit of each model and to identify the best random effect (error structure) model. We report the results of the model with the lowest AIC value for each growth parameter. In every case, the chosen model fit a random slope and a random intercept value to the predicted growth curve. This model allows each nestling to be a different size at hatching and to grow at a unique rate for each individual.

Immune responses were compared between fumigated and control nests for adult females, adult males, and nestlings using a two-way ANOVA with Bonferroni multiple comparison post-hoc tests ($\alpha < 0.05$). We used a Spearman correlation to look at the relationship between parent female immune responses and parasite abundance in control nests.

Behaviors were compared between treatments using a Wilcoxon matched-pairs sign rank test (data could not be transformed to achieve normality). Due to small sample sizes for behavioral comparison and large degrees of variation within treatments, we also report the effect size and 95% confidence interval for some comparisons of behavior. Effect size is defined as the difference between the means of each treatment (Nakagawa & Cuthill, 2007, Nakagawa, 2004). An F test to compare variances is also reported for some comparisons of behaviors between treatments. We used a Spearman correlation to test the relationship between nest sanitation behavior and parasite abundance in control nests.

Results

Parasite abundance

In 2009, no *P. downsi* were found in the seven fumigated nests, while the six control nests had a mean *P. downsi* abundance of 30.5 ± 7.53. In 2010, fumigated nests (n = 21 nests) had a mean *P. downsi* abundance of 0.23 ± 0.19, compared to control nests (n = 22 nests) which had 38.50 ± 5.13. Fumigated nests had significantly fewer *P. downsi* than control nests (t-test, $t = 7.40$, $p < 0.0001$).

Immune response

Philornis downsi-specific antibody responses (optical density, OD) differed significantly between parent females, parent males and nestlings (Two-way ANOVA, family status: $F_{2,107} = 97.42$, $p < 0.001$; Fig. 5.1). Female parents had greater *P. downsi*-specific antibody responses than male parents. Both parent females and parent males had greater responses than nestlings. There was a significant effect of treatment across parent females, parent males and nestlings (treatment: $F_{1,107} = 4.58$, $p = 0.034$) but no significant interaction (treatment*family status: $F_{2,107} = 1.72$, $p = 0.18$). Bonferroni post-hoc comparisons show that females in control nests had significantly greater *P. downsi*-specific antibody responses than females in fumigated nests ($t = 0.32$, $p < 0.05$). However, neither male nor nestling antibody responses differed significantly between treatments (males: $t = 1.01$, $p > 0.05$; nestlings: $t = 0.10$, $p > 0.05$).

There was a marginally non-significant negative correlation between female *P. downsi*-specific antibody response and parasite abundance within control nests (Spearman correlation, $r = -0.48$, $p = 0.06$, Fig. 5.2). Females with greater antibody responses tended to have nests with lower parasite abundance.

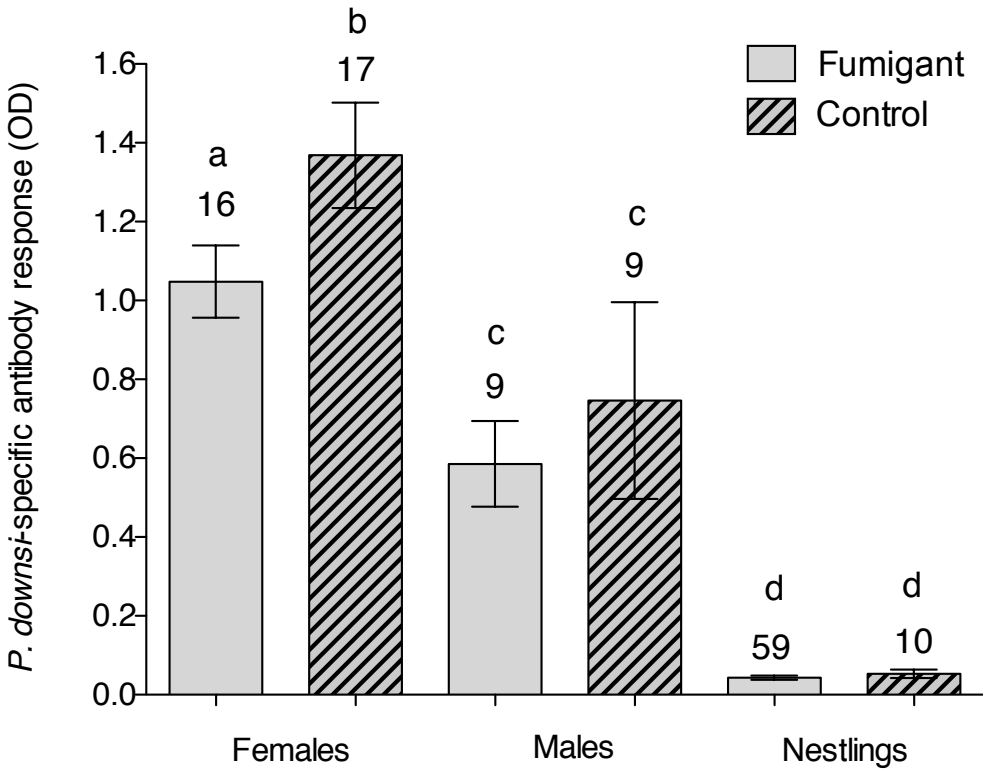


Figure 5.1. Mean (± SE) anti-*P. downsi* antibody responses (optical density, OD) of females, males, and nestlings from fumigated (gray bars) and control (hatched bars) nests. The number of individuals sampled is shown above each bar. Letters indicate groups that differ significantly using Bonferroni post-hoc comparisons between treatments.

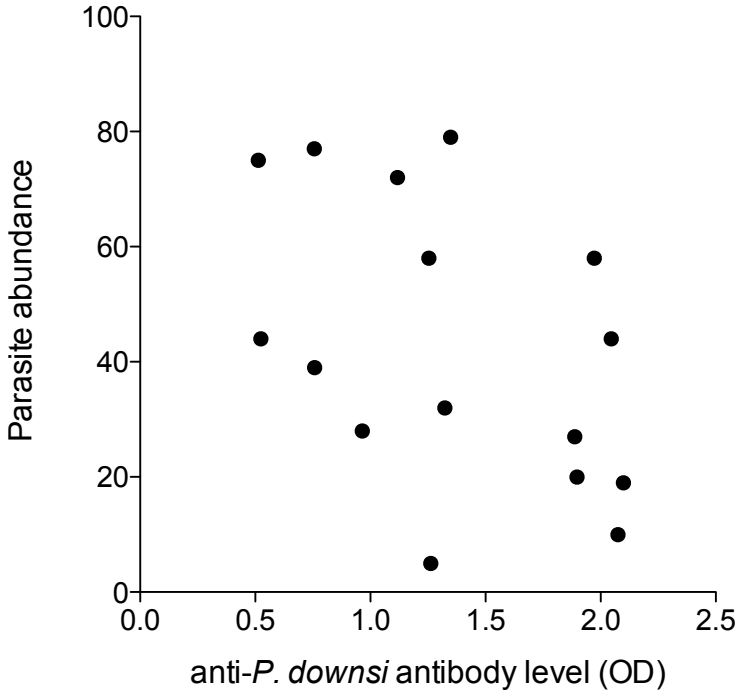


Figure 5.2. Relationship between adult female anti-*P. downsi* antibody response and *P. downsi* abundance within control nests. Females with greater antibody responses tended to have nests with lower parasite abundance.

Behavior

During the daytime, females in control nests spent 44.1 ± 6.6% of their time at the nest compared to females in fumigated nests that spent 56.1 ± 9.1% of their time at the nest. Females in fumigated nests did not differ significantly in time in attendance at the nest compared to females in control nests ($W = -27.0$, $p = 0.13$). At night, all females in both treatments were present at the nest for the entire duration of the video recordings.

During the day, females in control nests spent 36.5 ± 9.0% of their time at the nest brooding nestlings compared to 64.0 ± 8.6% by females in fumigated nests. Females in control nests spent significantly less time at the nest brooding their nestlings than females in fumigated nests ($W = 34$, $p = 0.02$, effect size = 27.5%, 95% CI = 1.1% to 54%, Fig. 5.3A). At night, females in control nests spent 60.3 ± 18.3% of their time at the nest brooding compared to 92.0 ± 1.5% by females in fumigated nests. Females did not differ significantly in the amount of time they spent brooding nestlings ($W = 11$, $p = 0.19$, effect size = 31.6%, 95% CI = -10.6% to 73.9%, Fig. 5.3B); however, females in control nests were significantly more variable in amount of time spent brooding compared to females in fumigated nests ($F_{4,4} = 143.7$, $p < 0.001$).

During the day, females in control nests spent 10.5 ± 3.5% of their time at the nest performing nest sanitation behavior compared to 5.9 ± 1.7% by females in fumigated nests. Females in fumigated and control nests did not differ significantly in the amount of time they performed nest sanitation behavior while at the nest ($W = -9.0$, $p = 0.65$, effect size = 4.5%, 95% CI = 12.8% to -3.7%, Fig. 5.3C). At night, females in control nests spent 19.6 ± 6.8% of their time at the nest performing nest sanitation compared to 6.6 ± 1.8% by females in fumigated nests. Females again did not differ significantly in

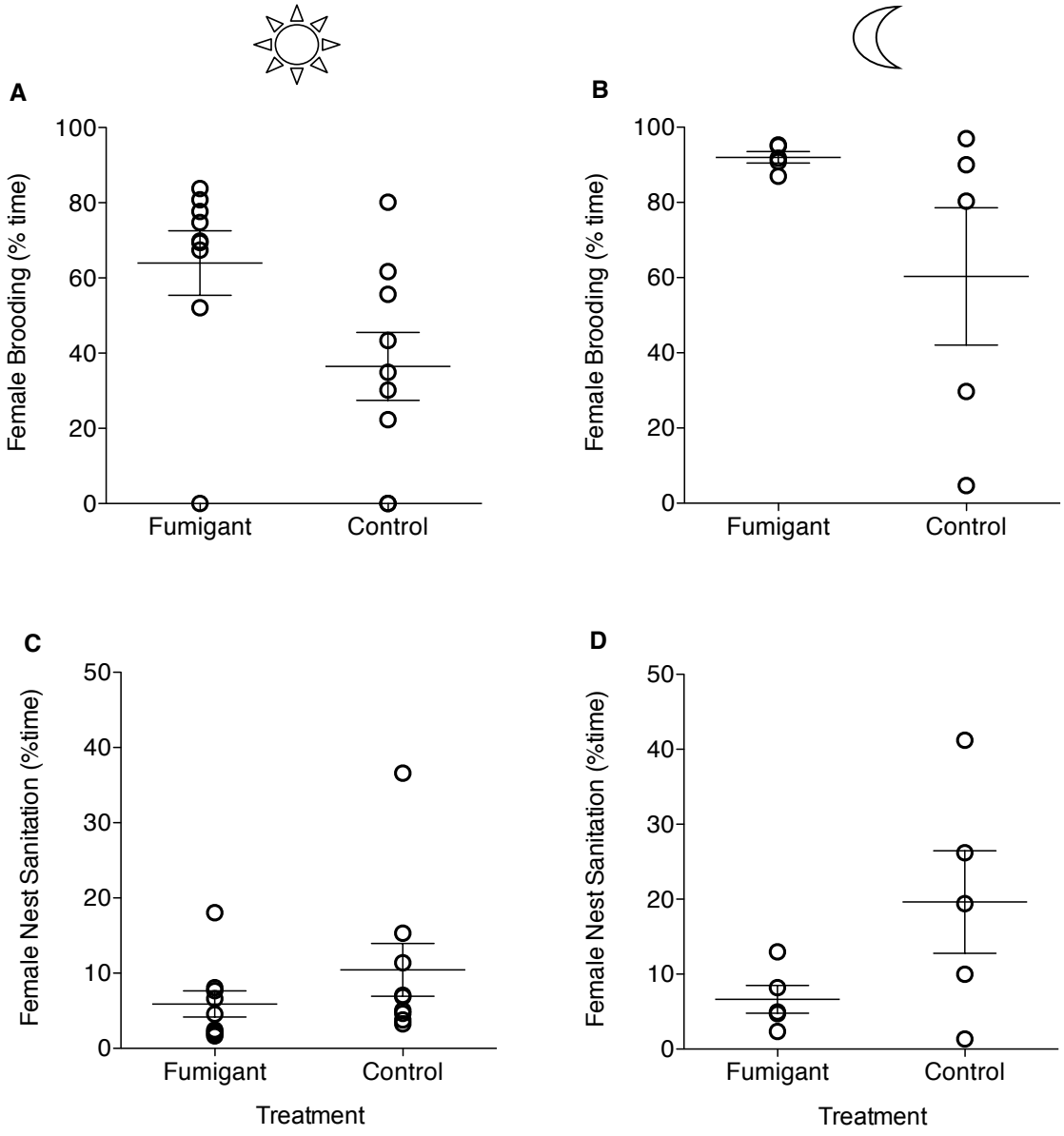


Figure 5.3. Differences in female brooding (A, B) and nest sanitation (C, D) behaviors between fumigated and control nests. Behaviors are presented as a percentage of the time females were at the nest. Daytime behaviors are in the left-hand column and nighttime behaviors are in the right hand column. Bars show the mean ± S.E.

the amount of time they engaged in nest sanitation behavior between treatments (W = -13, p = 0.13, effect size = 13.0%, 95% CI = 29.3% to -3.3%, Fig. 5.3D). However, females in control nests showed significantly more variation in the amount of time they performed nest sanitation at night compared to females in fumigated nests (F test for variances, $F_{4,4} = 13.81$, p = 0.03). There was not a significant correlation between parasite abundance and the amount of time females spent at the nest performing nest sanitation during the day (Spearman correlation, r = 0.32, p = 0.41) or at night (r = 0.60, p = 0.35).

Parents spent 3.1 ± 0.4% of their time feeding nestlings in control nests compared to 4.4 ± 0.6% feeding nestlings in fumigated nests. Parents in fumigated and control nests differed significantly in the amount of time spent feeding nestlings, though the difference was small (W = 35.0, p = 0.04).

During the day, nestlings in control nests were agitated 1.5 ± 0.6% of the observed time compared to 0.2 ± 0.1% by nestlings in fumigated nests. There was a marginally non-significant difference between treatments in the amount of time nestlings appeared agitated (W = -27, p = 0.07). Nestlings in control nests were significantly more variable in the amount of time they were agitated than nestlings in fumigated nests ($F_{8,8} = 70.54$, p < 0.0001).

Instances of allopreening were observed too rarely in nests of either treatment to compare statistically. One female in a fumigated nest and four females in control nests were observed allopreening nestlings. However, of the females that did allopreen nestlings, the behavior consisted of less than 2% of their total time at the nest. No observations of allopreening were recorded at night in either treatment. We observed

nestlings preening themselves in only three nests, consisting of less than 1% of the total observed time. Nestlings younger than 5 days are still relatively uncoordinated and without plumage which likely contributed to the rare observations of preening and allopreening in our study.

Nestling growth

There was a significant interaction between treatment and age on nestling mass, tarsus length and outermost primary feather length (Table 5.1, Fig. 5.4). Nestlings in control nests grew significantly slower than nestlings in fumigated nests, gaining less mass, and having shorter tarsi and outermost primary feathers. Table 5.1 summarizes the coefficients of the best model for each growth parameter.

There was a significant effect of treatment on the intercept values for both mass and tarsus. The respective negative values suggest that nestlings in fumigated nests were smaller at the time of hatching than nestlings in control nests. However, these estimated intercept values are an artifact of the significantly different slope values and do not reflect actual nestling size at hatching.

Hematocrit

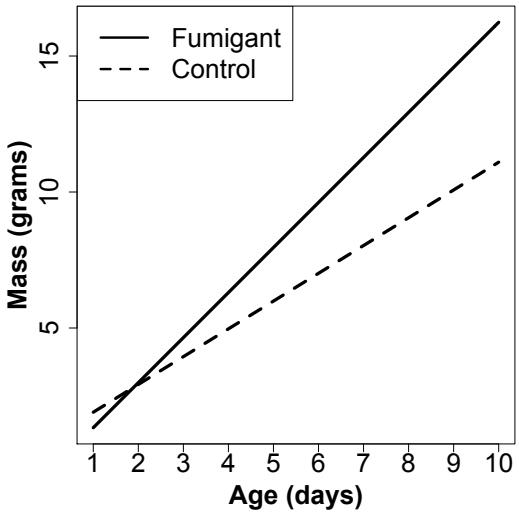
Hematocrit values did not differ significantly between parent females from fumigated (46.79 ± 1.03%, n = 15) and control nests (47.07 ± 0.92%, n =14) (Mann-Whitney, U = 102, p = 0.91). Hematocrit values also did not differ significantly between parent males from fumigated (50.44 ± 1.06%, n = 9) and control nests (49.50 ± 1.21%, n = 8) (U = 31.5, p = 0.70).

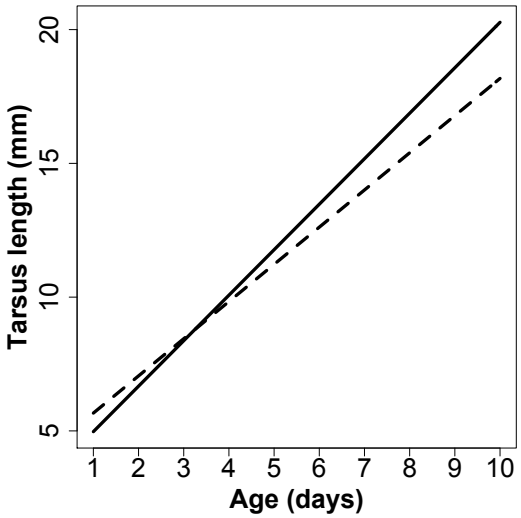
To avoid pseudoreplication, we compared nestling hematocrit values between treatments by using a mean brood value for all nestlings within a nest. Nestlings in

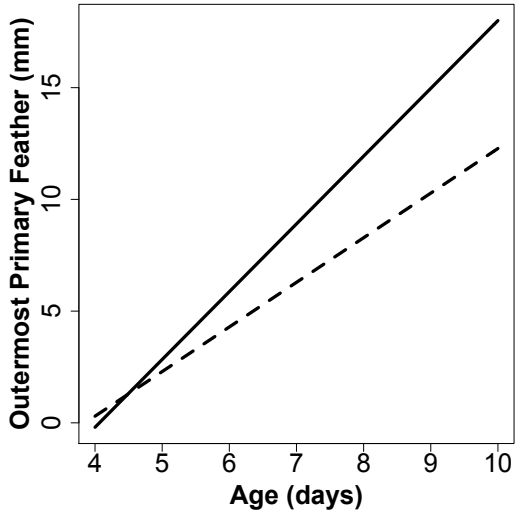
Table 5.1. Linear mixed-effects model to compare growth parameters of nestlings in fumigated and control nests. Individual nestling identity is treated as a random effect clustered by nest. There was a significant effect of treatment and age on each growth parameter measured such that nestlings in fumigated nests grew significantly faster than nestlings in control nests. Nestlings in fumigated nests gained 0.66 g more per day than nestlings in control nests. Similarly, nestlings in fumigated nests had tarsi that grew 0.35 mm more per day and had outermost primary feathers that grew 1.00 mm more per day than nestlings in control nests.

Model = Growth parameter ~ b_0 + b_1*Treatment + b_2*Age + b_3*Treatment*Age					
Growth parameter	**Fixed Effect**	**Coefficient ± SE of Fixed Effect**	**DF**	**t-value**	**p-value**
Mass (g)	Treatment	-0.60 ± 0.16	132	-3.82	< 0.001
	Age	1.01 ± 0.08	268	12.60	< 0.0001
	Treatment*Age	0.66 ± 0.10	268	6.79	< 0.0001
Tarsus (mm)	Treatment	-0.70 ± 0.19	124	-3.74	< 0.01
	Age	1.35 ± 0.05	210	26.93	< 0.0001
	Treatment*Age	0.35 ± 0.06	210	6.12	< 0.0001
Primary feather (mm)	Treatment	-0.54 ± 0.32	101	-1.71	0.09
	Age	1.99 ± 0.18	111	11.15	< 0.0001
	Treatment*Age	1.00 ± 0.19	111	5.15	< 0.0001

Figure 5.4. Results of linear effects mixed models used to predict the effect of nest treatment and nestling age on (A) mass, (B) tarsus length, and (C) outermost primary feather length. Nestlings in fumigated nests (black line) grew more quickly than nestlings in control nests (dashed line). Primary feathers are not present in nestling medium ground finches at hatching; therefore, plot (C) shows the model predictions based on data collected starting when nestlings were 4 days old. The predictions for each growth parameter show that nestlings in fumigated nests grew faster than nestlings in control nests.


Fumigant
Control
Mass (grams)
5
10
15
1 2 3 4 5 6 7 8 9 10
Age (days)


Tarsus length (mm)
5
10
15
20
1 2 3 4 5 6 7 8 9 10
Age (days)


Outermost Primary Feather (mm)
0
5
10
15
4 5 6 7 8 9 10
Age (days)

control nests (34.61 ± 0.61%, n = 4 nests) had significantly lower hematocrit than nestlings in fumigated nests (37.50 ± 0.81%, n = 21 nests) (U = 18, p = 0.04, Fig. 5.5). The small number of control nests is a result of rapid nestling mortality in this treatment group; many control nests had completely failed by the time nestlings would have sampled for blood.

Nestling survival

In 2010, 21 of 21 (100%) fumigated nests had at least one nestling survive to 9 days of age, compared to 2 of 22 (9%) control nests. Fumigated nests had significantly more nestlings survive to 9 days of age compared to control nests (Fisher's Exact, $p < 0.0001$). Sixty-five of 74 (88%) nestlings in fumigated nests survived to 9 days of age compared to 3 of 62 (5%) nestlings in control nests. Significantly more nestlings survived to 9 days of age in fumigated nests than nestlings in control nests ($p < 0.0001$).

Fledging success

In 2009, one of seven (14%) fumigated nests successfully fledged at least one offspring compared to two of six (33%) control nests. Fledging success did not differ significantly between fumigated and control nests, though the number of nests to produce fledglings was extremely low in both treatments ($p = 0.56$). Two of 15 (13%) nestlings in fumigated nests successfully fledged compared to 4 of 14 (29%) nestlings in control nests ($p = 0.34$).

In 2010, 21 of 21 fumigated nests (100%) successful fledged at least one offspring while none of the 22 (0%) control nests did so. Significantly more fumigated nests fledged at least one offspring compared to control nests ($p < 0.0001$, Fig. 5.6A). In

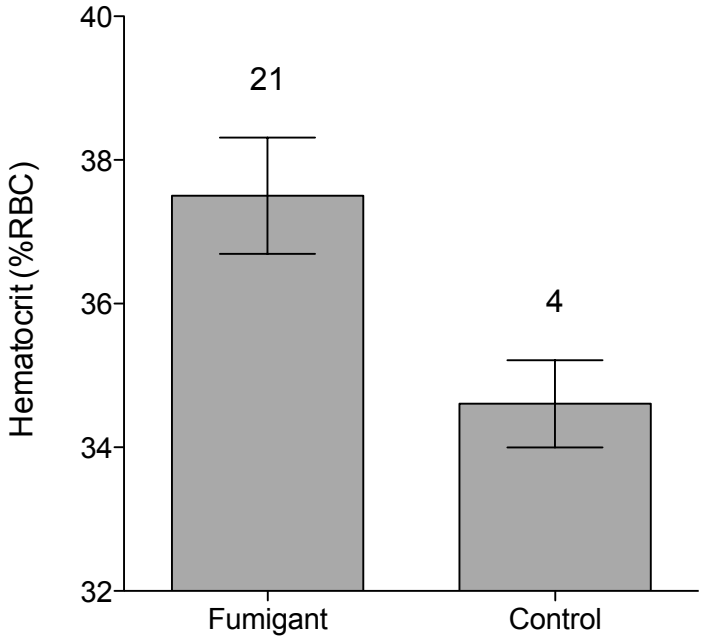
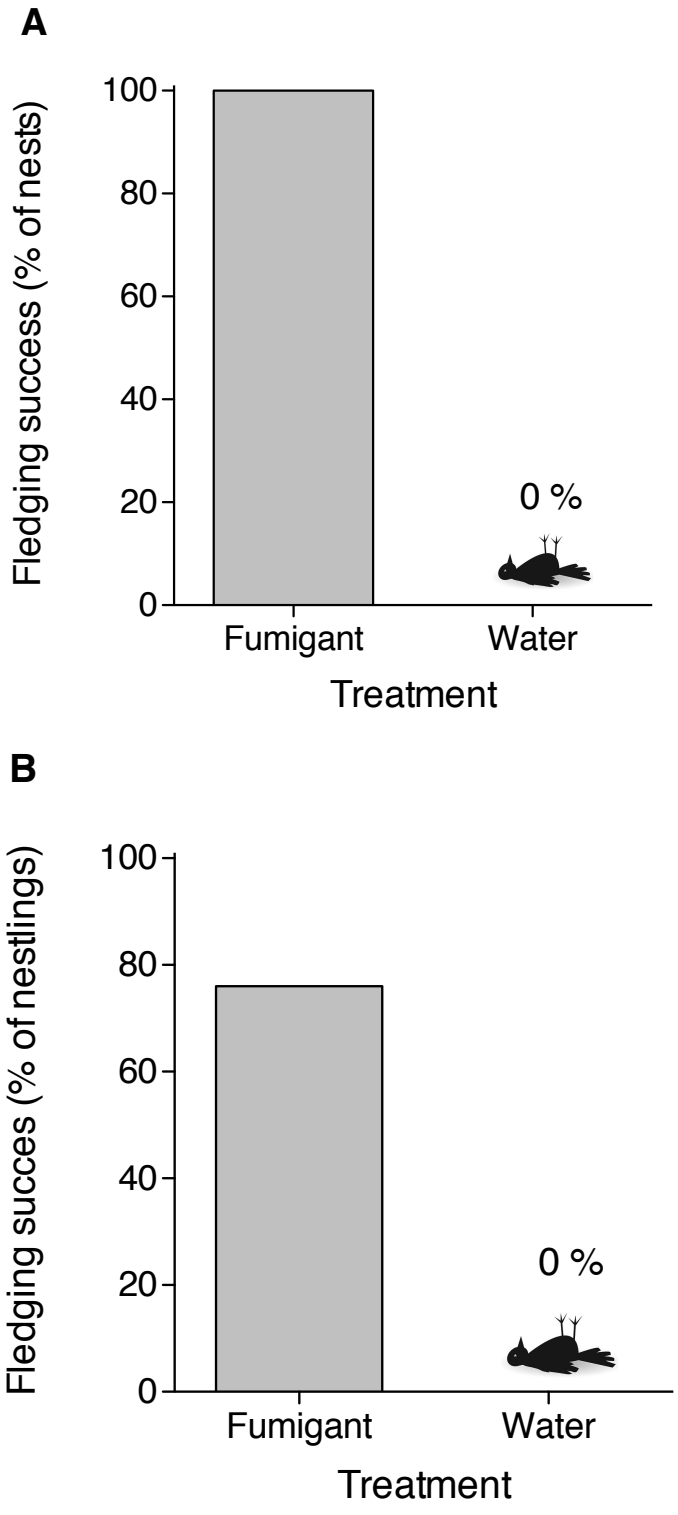


Figure 5.5. Mean (± SE) hematocrit values for nestlings in fumigated and control nests. Hematocrit is the proportion of red blood cells (RBC) out of the total blood volume. Values for nestlings within a single nest were averaged to avoid pseudoreplication. Numbers above each bar indicate the number of nests sampled. Nestlings in fumigated nest had significantly higher hematocrit than nestlings in control nests.

Figure 5.6. Effect of nest treatment on host fledging success in 2010. (A) The proportion of nests to successfully fledge at least one offspring in fumigated (21 of 21 nests) and control (0 of 22 nests) treatments. (B) The proportion of nestlings to successfully fledge from fumigated (56 of 74 nestlings) and control (0 of 62 nestlings) nests.


A
Fledging success (% of nests)
100
80
60
40
20
0
0 %
Fumigant
Water
Treatment
B
Fledging succes (% of nestlings)
100
80
60
40
20
0
0 %
Fumigant
Water
Treatment

fumigated nests 56 of 74 (76%) nestlings successfully fledged compared to 0 of 62 nestlings from control nests ($p < 0.0001$, Fig. 5.6B).

Discussion

Our study investigated the presence and efficacy of potential defenses by medium ground finches against the nest parasite, *P. downsi*. We used fumigant to experimentally manipulate *P. downsi* abundance in nests to compare nestling growth, condition, and survival, fledging success between fumigated and control nests. *P. downsi* significantly reduced nestling growth, hematocrit, and survival to 9 days of age. While limited sample size in 2009 showed inconclusive results, the impact of *P. downsi* on fledging success in 2010 was severe, with 100% mortality in control nests. To investigate potential defense mechanisms, we compared adult and nestling immune responses and behaviors between fumigated and control nests. Adult medium ground finches mount *P. downsi*-specific antibody-mediated immune responses. Within control nests, females with greater *P. downsi*-specific antibody responses tended to be in nests with lower *P. downsi* abundance. Nestlings did not mount immune responses above the detectable threshold of our immuno-assay. Parents of fumigated nests fed their nestlings more often than parents of control nests. Females spent more time brooding nestlings in fumigated nests than control nests. However, females did not differ significantly between treatments in the amount of time they performed nest sanitation behavior, nor did nest sanitation behavior correlate with *P. downsi* abundance in nests. Nestlings in fumigated nests tended to be less agitated than nestlings in control nests. Preening, a common defensive behavior by birds against ectoparasites, was rarely observed in young nestlings (all nestlings observed were less than 6 days old).

In 2009, we tested the effectiveness of using fumigant to control *P. downsi* abundance in the nests of medium ground finches. The fumigant completely eliminated *P. downsi* from the nests to which it was applied. We observed very few breeding attempts in 2009 due to dry conditions, precluding a conclusive comparison of fledging success between treatments. Only three nests from both treatments successfully fledged offspring. Fledglings from one of the control nests were found on the ground immediately below the nest and were extremely lethargic when approached. Fledglings from the fumigated nests were observed begging for food in a tree ~ 50 m from the nest. As shown in the growth model presented herein, even nestlings able to survive to near fledging age in control nests would be significantly smaller than nestlings of the same age from fumigated nests. Size at fledging is a reliable indicator of post-fledging survival (Martin, 1987, Arendt, 1985), suggesting that fledglings from parasitized nests would be less likely to survive.

Previous studies have demonstrated significant effects of *P. downsi* on nestling growth and host fledging success (reviewed in Koop et al., 2011), but to our knowledge, none were as severe as those reported here. *P. downsi* significantly reduced nestling growth and hematocrit. In 2010, more than 80% of nestlings had died by 5 days of age. As a measure of fledging success, we observed complete nest failure in control nests compared to at least one successful fledgling from every fumigated nest. Such high levels of nestling mortality should result in strong selection for effective defense mechanisms.

Our results show that adult medium ground finches are able to produce *P. downsi*-specific antibodies, consistent with Huber et al. (2010), and that the magnitude of these

antibody responses reflects *P. downsi* abundance in nests. Effective antibody-mediated immune responses can increase the speed and intensity of an inflammatory response in hosts (Owen et al., 2010). Parasites feeding on inflamed regions of the host have a more difficult time accessing capillary beds and successfully feeding. Owen et al. (2009) showed that inflammatory responses by chickens (*Gallus gallus domesticus*) impaired the feeding ability of northern fowl mites (*Ornithonyssus sylviarum*) leading to a dramatic decrease in parasite abundance on the host. Our results suggest that females with stronger antibody responses had nests with lower *P. downsi* abundance. This relationship suggests that host immune response decreases parasite fitness. *P. downsi* are able to move between and feed on adults and nestlings within the nest. Since adults and nestlings do not have equal immune responses, larvae feeding solely on nestlings likely add variation to the observed relationship between parent female antibody response and parasite abundance. Nonetheless, a strong antibody-mediated immune response by females could infer a large fitness benefit if it decreases parasite abundance in the nest.

Immune responses in nestlings, produced endogenously or by maternal transfer of antibodies, can act as an effective defense mechanism against ectoparasites in the nest (Grindstaff, 2008, King et al., 2010, Boulinier & Staszewski, 2008). We did not find detectable quantities of *P. downsi*-specific antibodies in 5-day old nestlings. The half-life of maternally transferred antibodies in passerine nestling plasma is approximately 2 days. Nestlings are able to produce endogenous antibodies within 3-4 days of age (King et al., 2010). We took a blood sample from nestlings at 5 days of age to allow body size to reach a point where blood sampling was less likely to have confounding effects on survival. Our results show that 5-day old nestlings do not have *P. downsi*-specific

antibodies (endogenously produced or maternally transferred) above the detectable threshold of our immuno-assay. Our results provide no evidence that nestlings are able to immunologically defend themselves against *P. downsi*. Further work is needed to determine whether nestlings that survive beyond 5 days of age produce *P. downsi*-specific antibodies in relevant quantities.

In some birds, parents are able to alter their behavior such that they can negate the effects of parasitism on their nestlings. Several studies have found support for the food compensation hypothesis, which suggests that parents can increase food provisioning to parasitized nestlings to compensate for some of the energy lost to parasites (Tripet & Richner, 1997, Hurtrez-Bousses et al., 1998, Wesolowski, 2001). However, Christe et al. (1996a) suggest that parents may actually decrease food provisioning to the nest if nestlings are too weak to beg for food. In our study, parents spent less time feeding nestlings in control nests than those in fumigated nests. Nestlings began begging immediately after a parent arrived at the nest and were fed until begging ceased. Only nestlings older than six days were observed begging outside of the time when parents were actually feeding them. We were not able to compare parental food provisioning to older nestlings due to extensive mortality of nestlings in control nests. Our results suggest that in the early nestling period, decreased feeding may further exacerbate the effects of *P. downsi* on nestling survival.

Nestling condition may further be compromised by decreases in the quality of brooding provided to parasitized nestlings. While at the nest, females in control nests varied greatly in the amount of time they spent brooding nestlings both in the day and at night compared to females in fumigated nests. During the day, females in fumigated

nests spent significantly more time brooding than females in control nests. Disruptions in brooding were often characterized by females standing over nestlings. Nestlings frequently appeared agitated in control nests, which may explain disruptions in female brooding. At night, disruptions in brooding were often followed by episodes of nest sanitation, particularly in control nests. Disruptions in brooding may decrease the efficiency of heat transfer from the female to nestlings and may also decrease the quality of sleep for both adults and nestlings (Christe et al., 1996b). Additional energy required by parents or nestlings to compensate for these losses would likely result in poorer nestling condition. We did not detect a significant difference in nest sanitation behavior at night, though this may be due to small sample size.

In highly variable environments such as the Galapagos, life-history theory predicts that birds should invest heavily in current reproductive efforts as future reproduction is less certain (Ricklefs, 1977, Karell et al., 2009). Our data are consistent with this prediction; we did not find a significant difference between treatments in the amount of time females attended their nests. Even at night, when females in control nests appeared agitated, they did not leave the nestlings unattended. Only a single case of nest abandonment was observed in a parasitized nest (due to inclement weather and thus, not included in this study), and no cases were observed in fumigated nests. Females were even observed brooding dead nestlings over the course of a day before either removing them from the nest or, in the case of total brood failure, leaving the nest. Thus, medium ground finches do not abandon parasitized nestlings in favor of self-preservation or future reproductive attempts.

Nest sanitation behavior is commonly observed in breeding birds and is thought to have an antiparasitic function (Clayton et al., 2010). Female blue tits (*Parus caeruleus*) in nests parasitized by blowflies (*Protocalliphora*) increased nest sanitation behavior compared to parents in unparasitized nests (Hurtrez-Bousses et al., 2000). We did not observe significant differences in nest sanitation behavior between treatments. However, females in control nests varied significantly more in the amount of time spent performing nest sanitation than females in fumigated nests at night, when *P. downsi* are most active (O'Connor et al., 2010). The high degree of variation in this behavior suggests that females may respond to *P. downsi* presence in the nest by increasing nest sanitation behavior, though small sample size precludes a conclusive analysis.

We did not find a significant correlation between the amount of time adult females spent performing nest sanitation and *P. downsi* abundance in the nest. Late instar *P. downsi* are approximately one centimeter in length, which may allow them to expose their mouthparts and feed on nestlings while the remainder of their body stays largely embedded in the nest material. This strategic positioning may allow larva to quickly escape host nest sanitation behavior. While further studies are needed to understand escape behavior in *P. downsi*, our data suggest that nest sanitation behavior by parent finches is an ineffective defense against *P. downsi*.

In summary, we do not see evidence of effective defense mechanisms in the observed population of medium ground finches. Other populations or species may use different defense mechanisms from those observed in our study population. Some species of birds incorporate aromatic green vegetation into their nests supposedly to combat ectoparasites, such as mites and blowflies (Shutler & Campbell, 2007, Ontiveros

et al., 2008, Clark, 1990). Feathers lining the inside of nests serve primarily as an insulating layer but may also act as a protective barrier between nestlings and larvae living in the bottom of the nest (Winkler, 1993). Finally, females may be able to transfer hormones, carotenoids or other immune-active substances to eggs such that nestlings are better able to defend themselves against parasites in the nest (Ewen et al., 2009, Tschirren et al., 2009, Saino et al., 2002, Saino et al., 2003). Further work is needed to investigate variation in the presence and efficacy of host defenses against *P. downsi* in other populations and species of Darwin's finches.

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CHAPTER 6

THE DEMISE OF DARWIN'S FINCHES? A MODELING APPROACH TO ASSESS THE IMPACT OF AN INTRODUCED PARASITE ON HOST POPULATION VIABILITY

Abstract

Introduced parasites and pathogens present one of the greatest threats to naïve host populations, especially those on islands. *Philornis downsi*, an obligate nest parasite of birds recently introduced to the Galapagos Islands has been implicated in the severe population declines of several endangered Darwin's finch species. We use data from a three-year experimental study of the effects of *P. downsi* on medium ground finch (*Geospiza fortis*) fitness to model population viability. Extremely high levels of host mortality (100% in some years) are the impetus for the creation of a model to predict population viability of medium ground finches. The parameters of the model are based primarily on our own data but also incorporate previously published studies on adult and fledgling annual survival. Under all iterations of the model medium ground finches on the island of Santa Cruz are predicted to go extinct within the next half-century. Medium ground finches are one of the most abundant Darwin's finch species; thus, our results demonstrate the potentially devastating effect this fly may have on non-native hosts. We discuss the urgent need for additional research to support and redefine the parameters of

our model. We also discuss conservation efforts currently underway to control *P. downsi* populations across the archipelago.

Introduction

Introduced parasites and pathogens are now recognized as an increasing global threat to naïve wildlife host populations. Parasite or pathogen driven extinction has been documented across several taxa, such as the Polynesian snail due to a protozoan (Cunningham & Daszak, 1998), multiple amphibian species due to Chytridiomycosis (Berger et al., 1998, Lips et al., 2006), and Hawaiian honeycreeper species due to the introduction of avian malaria (van Riper et al., 1986, Warner, 1968). Also, the introduction of crustacean lice to wild pink salmon from farm raised salmon has caused severe declines in populations that could lead to local extinction (Krkosek et al., 2007). Unfortunately, many of these threats went unnoticed until host populations began to decline severely or had disappeared (McCallum & Dobson, 1995).

Small host populations, such as those on islands, are susceptible to problems of low genetic diversity or catastrophic environmental events that can lead to severe population declines and even local extinction. The introduction of parasites and pathogens to island populations of hosts can also have severely detrimental effects if the hosts they infest are not able to defend themselves (Cleaveland et al., 2002). Hosts and parasites that co-evolve over time have adaptations that allow hosts to defend themselves and parasites to escape host defenses, leading to stable population dynamics. In contrast, parasites introduced to naïve host populations can cause host populations to decline severely before hosts have time to evolve effective defenses.

In the Galapagos archipelago, no endemic bird species have gone extinct over recorded history (Parker et al., 2006). However, with the human population on the islands growing at 4% annually (Watkins & Cruz, 2007), introduced parasites and pathogens are a growing concern. An example of recent concern is the introduced fly, *Philornis downsi*, a hematophagous nest parasite of birds (Couri, 1985). Adult flies lay their eggs in the nest material or nostrils of nestlings. The eggs hatch and the larvae then complete three instars over 3-11 days. The larvae are parasitic and chew through the skin of nestling birds and consume the secreted blood and fluids. The larvae then pupate in the nesting material and emerge as adult flies 7-14 days later (Fessl et al., 2006, Dodge, 1971). The adult flies are nonparasitic and feed on decaying, organic matter. Aside from the basic life cycle, relatively little is known about the ecology of *P. downsi* (Dudaniec & Kleindorfer, 2006).

P. downsi was first noticed in the nests of birds in 1997 (Fessl & Tebbich, 2002). Since this observation, *P. downsi* has been documented on 11 of the 13 major islands (Wiedenfeld et al., 2007), and in the nests of 14 species of land birds, including 9 species of Darwin's finches (Fessl & Tebbich, 2002, Fessl et al., 2010, O'Connor et al., 2009). *P. downsi* has been implicated in the decline of three species of Darwin's finches including the critically endangered mangrove finch (*Camarhynchus heliobates*) on Isabela Island (Dvorak et al., 2004), as well as the medium tree finch (*Camarhynchus pauper*) and the warbler finch (*Certhidea fusca*) on Floreana Island (O'Connor et al., 2009, Grant et al., 2005). Recent observed declines in vegetarian finch (*Platyspiza crassirostris*) and vermilion flycatcher (*Pyrocephalus rubinus*) populations on Floreana island may also be

the result of *P. downsi* parasitism, in addition to habitat degradation (O'Connor et al., 2010c).

The mangrove finch is now one of the rarest birds in the world (Fessl et al., 2010). Less than 100 individuals exist across three populations on the island of Isabela. Male song has diverged between the remaining populations of mangrove finches, decreasing the likelihood that interpopulation breeding will occur (Brumm et al., 2010). Captive breeding programs have also been largely unsuccessful. Fessl et al. (2010) used a population viability model (Vortex v. 9.92) to assess the impact of high and low-intensity rat control on mangrove finch populations. The authors also tested the predictions of their model based on the observed impact of *P. downsi* on mangrove finch nesting success. The small population size of mangrove finches precludes the ability to perform rigorous experimental manipulations on the effects of *P. downsi* on mangrove finch fitness. Nests found with dead nestlings inside were considered to have failed due to parasitism (11% additional mortality due to *P. downsi* parasitism). By these parameter estimates, the model predicted that mangrove finch populations are expected to recover under conditions of high-intensity rat and fly control. However, the assumed impact of *P. downsi* on mangrove finch reproductive success is likely an underestimate since females remove dead nestlings from nests (Koop, pers. obs.). Rapid nestling mortality, within several days of hatching, could have also been misidentified as nest abandonment or inactivity, further underestimating the impact of *P. downsi*.

Early observational and correlational studies suggested that *P. downsi* negatively affects fledging success in several species of Darwin's finches (reviewed in Koop et al., 2011). Medium ground finches exist on Santa Cruz in large numbers and have served as

a model species for experimental studies of the impact of *P. downsi* on Darwin's finches. More recently, we performed a 3-year study on the impact of *P*. *downsi* on medium ground finches by experimentally manipulating parasite abundance in the nests. Our study took place January-April, 2008-2010 at El Garrapatero on the island of Santa Cruz. Briefly, medium ground finch nests were randomly divided into treated or control groups. Nests were treated with nylon liners (in 2008) or a 1% pyrethrin solution (in 2009 and 2010) to reduce the number of *P. downsi* in nests . Nests in the control group were sprayed with water or left unlined. Liners and pyrethrin were effective at significantly reducing *P. downsi* abundance in nests. We then monitored fledging success for the different treatment groups. The impact of *P. downsi* parasitism on fledging success over the 3-year study is summarized in Table 6.1.

In 2008 and 2010, nests with reduced *P. downsi* abundance had significantly greater fledgling success than parasitized nests. Both years were marked by extremely severe nestling mortality in control nests. In 2008, only a single control nest (of 24 nests) produced fledglings that were resighted after leaving the nest compared to 8 of 24 treated nests (33%). The contrast was even more striking in 2010, when no control nests produced fledglings (0 of 21 nests), compared to 100% of treated nests (22 of 22 nests) producing at least one fledgling. In 2009, there was not a significant difference between control and treated nests; dry conditions severely limited overall breeding that year (80% of nests failed to produce fledglings across both treatments).

The severe, but variable, effects of *P. downsi* on the fledging success of Darwin's finches are the impetus for this paper. We use a simple model to assess the impact of

Table 6.1. Summary of results from three-year study on the effects of *P. downsi* on medium ground finch fitness. Measurements of parasite abundance and prevalence, proportion of nests that fledged young, and average number of fledglings for treated and control nests during years 2008, 2009, and 2010. In 2008, nests were treated with nylon liners to reduce parasite abundance in nests while control nests were left unlined. In 2009 and 2010, treated nests were sprayed with a fumigant to eliminate parasites in nests while control nests were treated with water. Parasite abundance is the mean number of *P. downsi* in nests. Parasite intensity is the mean number of parasites per infested nest. Parasite prevalence is the number of nests infested as a proportion of the total number of nests observed. The proportion of nests with fledglings was calculated as the number of nests to fledge at least one offspring out of the total number of nests. The proportion of nests with nestlings that survived to at least 9 days of age is also shown in parentheses. The mean number of fledglings per nest is calculated as a proportion of the number of offspring that fledged out of the total number of nests. The mean number of nestlings that survived to at least 9 days of age per nest is shown in parentheses. Data are from Koop et al. 2011 and Koop et al. (in prep).

	Parasite prevalence	**Mean parasite abundance**	**Mean parasite intensity**	**Proportion of nests with fledglings**	**Mean # of fledglings per nest**
2008 Treated	0.79 (19 of 24 nests)	21.8	27.5	0.33 (0.50)	0.79 (1.08)
Control	0.96 (23 of 24 nests)	37.5	39.13	0.04 (0.29)	0.13 (0.54)
2009 Treated	0 (0 of 7 nests)	0	0	0.14 (0.57)	0.29 (1.00)
Control	0.83 (5 of 6 nests)	30.5	36.6	0.33 (0.33)	0.67 (0.83)
2010 Treated	0.10 (2 of 21 nests)	0.23	2.5	1.00 (1.00)	2.67 (3.10)
Control	1.00 (22 of 22 nests)	38.5	38.5	0 (0.09)	0 (0.14)

P. downsi on medium ground finch population viability. We have three main goals: 1) we model the effect this fly may have even on abundant host populations of Darwin's finches; 2) we estimate how effective conservation efforts must be to decrease the likelihood of extinction within the next 100 years; and 3) we provide recommendations concerning the type of further research needed on the effects of *P. downsi* for other populations and species of Darwin's finches.

Methods

Mathematical Model

We formulate a mathematical model based on the data summarized in Table 6.1. We only model the female population, since this group provides a strong indication of the viability of the entire population but allows for simplified parameter estimates. We also assume that the proportion of males and females in a given population is approximately equal, so that all females able to reproduce will be able to find a mate. A diagram of the model is shown in Figure 6.1.

The populations of steps (1), (2), (4), and (5) of Figure 6.1 are updated by binomial random variables as follows: $C_n = c_n * \text{Bin}(A_n, p_n)$, $I_n = \text{Bin}(C_n, q_n)$, $U_n = C_n - I_n$, and $A_{n+1} = \text{Bin}(F_n, s_n^F) + \text{Bin}(A_n, s_n^A)$. We assume the population is maintained at equilibrium by a density-dependent mortality rate that primarily affects the fledgling population. That is, the more adults in a given population, the lower the probability that a fledgling will survive and be recruited into the breeding population the following year. As a result, fledgling survival probability depends on the adult population in a logistic fashion as follows: $s_n^F = \max(s_n^0 (1-A_n/K), 0)$. Furthermore, since many females are capable of reproducing at one year of age, we assume all surviving female fledglings

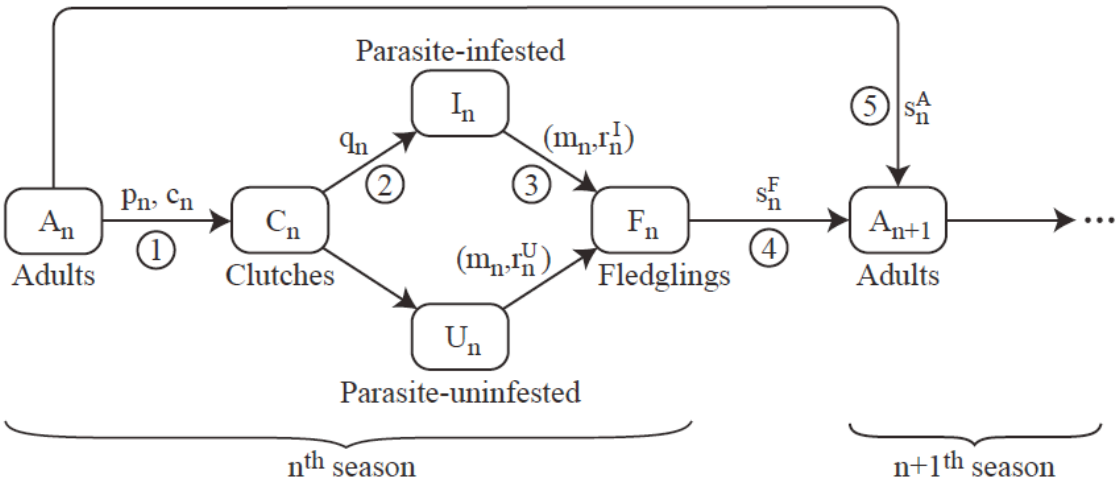

Figure 6.1. Diagram of model predicting annual reproductive fitness. The variable A_n is the number of female adults in the population at the beginning of year n. The model accounts for the following processes: (1) Each breeding season, a certain fraction of female adults breed (p_n) and produce a mean number of clutches per adult (c_n) for a cumulative number of C_n clutches in a breeding season. (2) A fraction (q_n) of clutches will become infested (I_n) by *P. downsi* or remain uninfested (U_n) in a given year. (3) Each infested (m_n, r_n^I) and uninfested (m_n, r_n^U) clutch produces a certain number of female fledglings (F_n) in a given year. (4) A fraction of female fledglings (s_n^F) from year n survive to become adults at the beginning of year $n + 1$. (5) A fraction of female adults (s_n^A) from year n survive to year $n + 1$.

become part of the potential adult breeding population A_{n+1} in the following year (Grant & Grant, 1992).

We model the number of fledglings produced in each clutch (step 3, Figure 6.1) in the following manner. During year *n*, each clutch is capable of producing a maximum number (m_n) of nestlings, some of which will successfully fledge. We let r_n^I and r_n^U be the probabilities that female nestlings fledge from infested and uninfested nests, respectively. As a result, the total number of fledglings produced by all clutches in year *n* is $F_n = \sum_{i=1}^{I_n} Bin(m_n, r_n^I) + \sum_{i=1}^{U_n} Bin(m_n, r_n^U)$. Due to a property of the binomial distribution, we can equivalently use the simpler expression $F_n = Bin(I_n m_n, r_n^I) + Bin(U_n m_n, r_n^U)$.

Nearly every interaction in the model is a function of the environmental conditions in a given year. Breeding and survival of finches is highly influenced by annual rainfall (Grant & Grant, 1992). In particular, wet years, characterized by relatively high rainfall January through April, lead to increased food supply and higher rates of breeding and survival. In contrast dry, or drought, years lead to a scarcity of resources and lower rates of breeding and survival. To a reasonable approximation, each year has this binary classification in terms of finch breeding and survival (Gibbs & Grant, 1987, Grant & Grant, 1992). As a result, in the model, we characterize each year (*n*) as being either wet or dry and do not model precise rainfall.

Parameter Estimates

Table 6.2 summarizes parameter estimates used in the model to predict population viability over time. In 2008, 2009, and 2010, the density of active nests found in the

Table 6.2. Parameter descriptions and estimates (from Koop et al., in press, in prep; Huber, 2008)

Parameter	Description	Estimate (wet year, dry year)
p_n	Probability of breeding	(0.95, 0.25)
c_n	Average number of clutches per breeding adult	(2,1)
q_n	Probability of infestation	(0.9, 0.8)
m_n	Max number of fledglings per clutch	(5, 3)
r_n^I	Probability of fledging successfully from an infested nest - Scenario 1: Estimate based on fledglings resighted - Scenario 2: Estimate based on nestlings surviving to day 9	 (0.0068, 0.11) (0.036, 0.14)
r_n^U	Probability of fledging successfully in an uninfested nest - Scenario 1: Estimate based on fledglings resighted - Scenario 2: Estimate based on nestlings surviving to day 9	 (0.27, 0.045) (0.31, 0.17)
s_n^F	Survival probability of fledglings	(0.75, 0.40)
K	Population carrying capacity	25000
s_n^A	Survival probability of adults	(0.9, 0.6)
	Frequency of wet years	1/3

surveyed area (1.5 x 1.5 km^2) was 21, 6, and 24 nests/km^2, respectively (Koop et al., in press, in prep). Thus, nesting density in dry years is approximately 1/4 that in wet years at our study site. We estimate that during dry years each adult female has a 25% chance (p_n = 0.25) of nesting, whereas during wet years each adult female has a 95% chance (p_n = 0.95) of nesting. As a result, p_n = 0.25 or 0.95 during dry and wet years, respectively. Based on the number of observed re-nesting events over our 3-year study period, we estimate that breeding adults produce an average of c_n = 1 clutch during dry years and c_n = 2 clutches in wet years.

We estimated the probability of a given nest becoming infested with *P. downsi* using prevalence data from control nests in our own studies (shown in Table 6.1), as well as that of Huber (2008) who surveyed *P. downsi* prevalence in the same population of medium ground finches at the same site over a 2-year period. Huber (2008) reported that *P. downsi* were present in 97% of medium ground finch nests in 2005, following a period of heavy rainfall, and in 64% of nests in 2006, which was an extremely dry year. Across our studies and Huber (2008), average prevalence was q_n = 0.74 in dry years and q_n = 0.98 in wet years.

We estimated fledging success for infested nests (m_n, r_n^I) and uninfested nests (m_n, r_n^U) with respect to wet and dry years. Estimates of fledging success are based on data for the mean number of fledglings produced by treated nests (2009, 2010) and control nests (2008, 2009, 2010). Treated nests from 2008 were excluded from these estimates because parasites were reduced, but not eliminated, in that year. These nests were not included as control nests due to the presence of liners in the nest. Based on our data, the maximum number of fledglings produced by any nest was five during a wet year

and three during a dry year. Thus, we set m_n = 5 or 3 during wet and dry years, respectively.

The model is run under independent scenarios for which fledging success is estimated according to two distinct operational definitions. The first scenario defines successful fledging conservatively as birds sighted and identified after leaving the nest (Koop et al., in press). The second scenario assumes that any nestling that survived to at least 9 days of age may have successfully fledged, despite not being sighted again after leaving the nest (Kleindorfer, 2007). We use this second scenario as a "best-case" estimate of fledging success to predict population viability.

Using maximum likelihood estimators to fit binomial distributions to the fledgling data under the first scenario (see appendix), we obtain $r_n^I = \frac{0.0682}{2m_n}$ and $r_n^U = \frac{2.672}{2m_n}$ for wet years, and $r_n^I = \frac{0.672}{2m_n}$ and $r_n^U = \frac{0.292}{2m_n}$ for dry years. (Note that we multiply each probability by a factor of ½, since we are only interested in counting female fledglings.) From these expressions, we obtain r_n^I = 0.0068 and r_n^U = 0.27 for wet years, and r_n^I = 0.11 and r_n^U = 0.045 for dry years. When we estimate the parameters based on the second operational definition of fledging success (nestlings that survived to at least 9 days of age), we obtain r_n^I = 0.036 and r_n^U = 0.31 for wet years, and r_n^I = 0.14 and r_n^U = 0.17 for dry years.

Our study did not explicitly follow fledgling survival or recruitment between years. A single fledgling from 2008 was recaptured in 2010 and colleagues recaptured four fledglings from 2010 in 2011. No fledglings from 2009 were recaptured in 2010 or 2011. All of the fledglings recaptured in subsequent years were from treated nests.

Based on these estimates, annual first year fledgling survival is < 20% for fledglings from treated nests and 0% for fledglings from control nests. These results are likely underestimates since we did not perform a mark-recapture study to estimate dispersal.

Thus, our estimates for these parameters rely on published studies of medium ground finch fledgling survival in populations on Daphne Major (Grant & Grant, 1992, Price & Grant, 1984). At present, *P. downsi* has not been found in the nests of any species of finches on Daphne Major. Of the five cohorts followed by Grant and Grant (1992) and Price and Grant (1984), ~45% of fledglings survived to the next year. Fledging success was extremely variable in these cohorts, therefore we use an average across years rather than defining separate probabilities for wet and dry years. Furthermore, larger islands such as Santa Cruz likely experience conditions that are somewhat buffered compared to those on Daphne Major. Therefore, we use a higher estimate of fledging first year survival in the model, such that $s^F = 0.55$.

Recapture rates of adults at our field site were consistently low (0-40%); these results may reflect high rates of emigration (Hendry et al., 2009, Koop, unpublished data). So again, we rely on estimates of adult survival from populations of medium ground finches on the island of Daphne Major, where emigration from the island is likely more rare. Approximately 60% of the adult population of medium ground finches on Daphne Major survive from one year to the next (averaged across wet and dry years) (Gibbs & Grant, 1987). Again, since conditions on Daphne Major may be more extreme than those on Santa Cruz, we use a slightly greater estimate of adult survival probability such that $s^A = 0.70$ in any given year.

We estimate that the population carrying capacity is $K = 25, 000$. Since Santa Cruz is 986 km^2, the carrying capacity translates to a population density of approximately 25 adult females per km^2. This density is calculated from the total number of females captured at our study site and averaged across years. Due to the approximation of this calculation, we ran several iterations of the model to estimate sensitivity to changes in population carrying capacity. Estimates of population viability were extremely robust to changes in initial carrying capacity (e.g., estimates of mean extinction times increased by < 10 years when population carrying capacity was increased by an order of magnitude to 250,000).

Of the last 46 years for which rainfall data have been collected, 67% were dry and 33% were wet years (http://www.darwinfoundation.org/datazone), so we model weather as a Bernoulli process with 1 out of 3 years being wet and the rest being dry.

Results

Based on 1,000 simulations of the model, we estimate the following mean times to extinction under the two operational definitions of fledging success. When fledgling is conservatively defined as those individuals sighted after leaving the nest, our model predicts extinction times for medium ground finch populations on the island of Santa Cruz within 42.0 ± 5.5 years (Figure 6.2). When we relax our definition of fledging success to include any nestling that survives to at least 9 days of age, the model predicts extinction times of 56.6 ± 9.8 years for populations of medium ground finches.

To determine the sensitivity of the model to parasite prevalence, we varied the parasite prevalence, q_n, during wet years from 0 to 100% and scaled the parasite prevalence during dry years accordingly. When parasite prevalence was reduced to

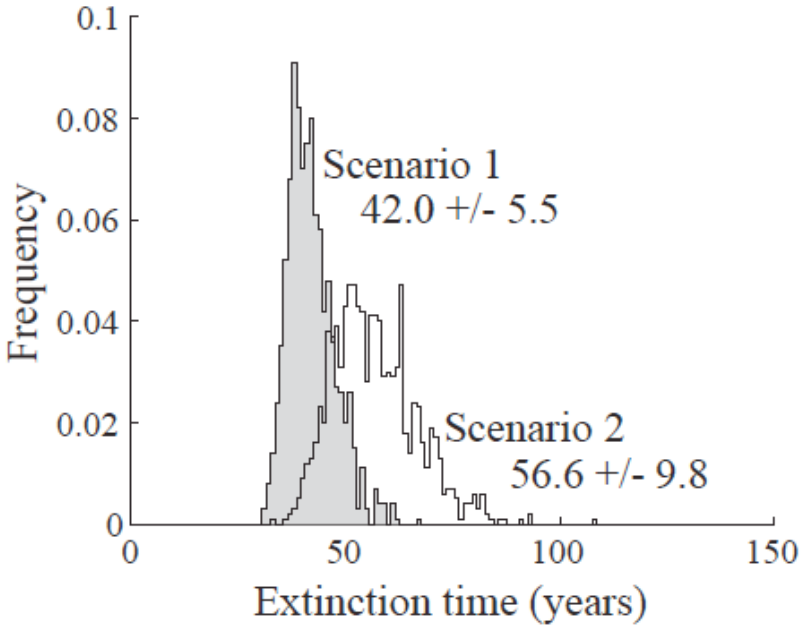

Figure 6.2. Distribution of extinction times over 1,000 model simulations for medium ground finches. Scenario 1 corresponds to estimates of fledging success based on the number of fledglings sighted after leaving the nest. Scenario 2 corresponds to estimates of fledgling success based on the number of nestling to survive to at least 9 days of age. The numbers shown are the model predictions for mean extinction times $\pm$ 1 standard deviation for medium ground finches on Santa Cruz Island.

~50% in wet years, the model predicted that medium ground finch populations would not go extinct within the next 100 years under the operational definition of fledglings sighted after leaving the nest. Based on estimates of fledging including all nestlings that survived to 9 days of age, prevalence of *P. downsi* needed to be reduced to ~75% in wet years for the population to persist beyond 100 years (Figure 6.3).

Discussion

Our model predicts that the medium ground finch on Santa Cruz island, which is currently very abundant, will decline to the point of extinction within the next half century. These estimates are the result of extremely low fledging success in parasitized nests, coupled with very high *P. downsi* prevalence. Our estimates of parasite prevalence (and related parasite intensities) are consistent with the reports of other studies across the archipelago (Dudaniec et al., 2006, Wiedenfeld et al., 2007, Fessl & Tebbich, 2002). *P. downsi* is now found in the nests of at least 14 species of birds across 11 islands, including both lowland and highland sites. Since its introduction, *P. downsi* has spread across the archipelago and into nearly every nesting habitat. This parasite poses a major threat to the Galapagos ecosystem and our model emphasizes the speed at which it may drive birds to extinction.

The severe effect of *P. downsi* on host fledging success was the impetus for the development of this model. One of the primary goals of this paper is to encourage further research aimed at improving the parameter estimates of our model and therefore improving the accuracy of its predictions. To this end, future work should focus on the following three areas: 1) Quantify the effects of *P. downsi* on other populations and species of birds throughout the archipelago; 2) Collect additional information on the

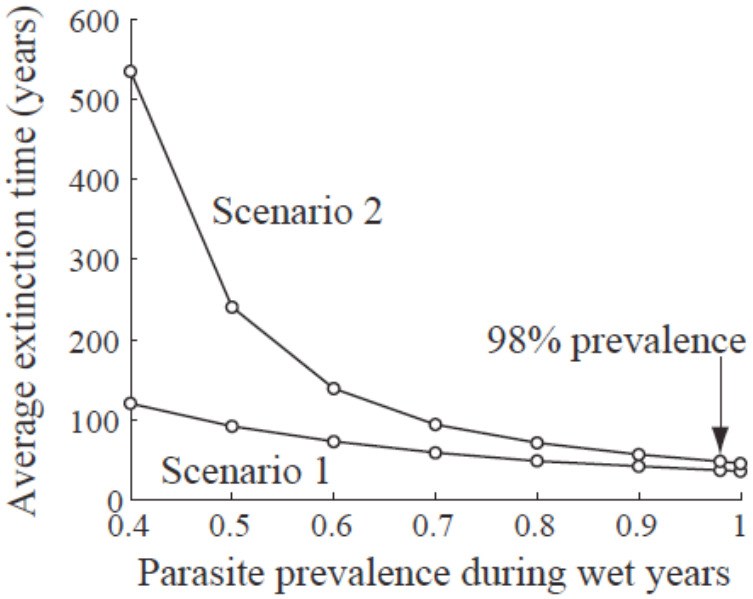

Figure 6.3. Mean extinction times as a function of parasite prevalence during wet years under both operational definitions of fledgling success. Under Scenario 1, where fledging success is defined only by fledglings that were sighted after leaving the nest, the predicted extinction times are relatively insensitive to changes in *P. downsi* prevalence. Under Scenario 2, where fledging success is defined by nestlings that survived to at least 9 days of age, predictions of extinction times significantly increase when *P. downsi* prevalence is below 70%.

biology of *P. downsi* relevant to the effects of abiotic factors and the mechanisms by which flies locate hosts; and 3) Test the design and efficacy of methods for controlling *P. downsi* populations.

Additional experimental studies are needed to investigate whether the observed effect on fledging success really does scale up to the level of populations and species. Experimental manipulations of parasite abundance in nests allow researchers to quantify direct effects of *P. downsi*. While correlational or observational studies can be informative, they cannot separate the effects of parasitism on fledging success from other variables such as rainfall and food availability (Grant & Boag, 1980, Grant, 1986, Gibbs & Grant, 1987). Thus, experimental manipulations should be performed whenever possible to delineate the effects of parasitism and provide comparable results across studies.

Our model assumes that all populations of medium ground finches on the island of Santa Cruz are affected similarly by *P. downsi*. This assumption is likely an oversimplification of the problem, though there are no data to support or deny this claim, further highlighting the need for additional studies (O'Connor et al., 2009, Dudaniec et al., 2006, O'Connor et al., 2010b, Huber, 2008, Koop et al., in press). *P. downsi* prevalence has been reported in studies of several populations of finches around Santa Cruz island and all are within the range estimated in our model . To date, there are no reports of populations of finches on Santa Cruz that are not infested by *P. downsi*. However, species or populations of finches that are more tolerant of *P. downsi* parasitism could alter the predictions of our model.

Host defenses may contribute to variation in the tolerance of different populations

or species of finches to *P. downsi* parasitism. Immunological and behavioral responses to *P. downsi* parasitism were completely ineffective in our study population of medium ground finches (Chapter 4). The ability to mount *P. downsi*-specific antibody immune responses was confirmed in six other species of Darwin's finches (Chapter 3), although further study is needed to determine whether these responses are effective. Populations with more abundant resources, such as those in the highlands, may be able to invest additional energy into immune responses, increasing their efficacy against *P. downsi*. O'Connor (2010b) observed nest sanitation behavior in small ground finches (*Geospiza fuliginosa*) and in Chapter 5 we observed similar changes in the parental behavior of medium ground finches. Further study is needed to investigate host defenses in other populations and species of finches in order to determine whether some populations may be more tolerant of *P. downsi*.

Seasonality effects of rainfall on finch breeding are expected to be more severe in lowland habitats than in highland habitats (Grant & Boag, 1980). As a result, finches in highland habitats may be able to breed for longer periods of time, even year-round, compared to finches in lowland habitats. Longer periods of breeding in highland populations could provide a source population of birds that are able to disperse into lowland populations. Population level surveys are needed to better understand movement of fledgling and adult finches on the island.

Abiotic factors, such as rainfall and temperature, also affect invertebrate populations, such as *P. downsi* (Goulson et al., 2005, Bennett & Whitworth, 1991). Consistent with our own observations, other studies show a relationship between *P. downsi* prevalence and rainfall. In general, dry conditions lead to lower parasite

prevalence (and intensity) while wet conditions support higher prevalence (Dudaniec et al., 2007, O'Connor et al., 2009, O'Connor et al., 2010a). Wiedenfled (2007) found higher *P. downsi* intensity on islands with humid highland habitat compared to islands with only arid, lowland habitat. Dry conditions may reduce *P. downsi* intensity such that finch reproductive success could increase even in parasitized nests. However, dry conditions are known to inhibit finch survival and reproduction for other reasons (Grant, 1986). Thus, the coupling of parasite and nonparasite effects may still limit reproductive success in years of reduced *P. downsi* intensity (Arendt, 1985).

The mechanism by which *P. downsi* locates host nests is currently unknown. Arendt (2000) suggested that adult flies may use light or odor cues to find host nests, although he was quick to point out that his results were inconclusive. Video observations in small and medium ground finch nests show adult *P. downsi* entering nests only when nestlings are present, never when there are only eggs in the nest (O'Connor et al., 2010b, Koop, pers. obs.). These observations suggest that flies may use nestling odors to locate nests. Adult flies may also use movement as a cue for finding nests, such as parents flushing from the nest entrance. Adult flies were observed entering nests within seconds of the brooding female finch leaving the nest. Interestingly, they were never observed entering the nests while the adult female was present (Koop, pers. obs.). Identifying the mechanisms by which flies locate hosts is important in predicting how changes in host density will affect parasite prevalence and intensity. Understanding these cues may also aid in the development of methods to control *P. downsi* populations.

Several methods are currently being considered for controlling *P. downsi* populations, although none are being used on a large scale. Traps designed to capture

adult flies offer a low-cost, low-maintenance option for reducing populations. However, attempts to capture adult flies using food bait (e.g., fruits, water, and syrups) have only been minimally successful (Fessl, pers. comm., Koop, pers. obs.). Pheromone traps use chemical cues to mimic potential mates. Collignon and Teale (2010) are investigating the chemical ecology of *P. downsi* to identify chemical receptors in adult flies. Female *P. downsi* are known to mate with multiple males (Dudaniec et al., 2010), which would increase the efficacy of a sterile insect program in which gravid females are exposed to experimentally sterilized males. This approach may be necessary to control *P. downsi* populations on large islands.

While our own studies show that the use of pyrethrin spray can eliminate *P. downsi* for the entire nesting period, this method is extremely labor intensive and therefore impractical in large populations of finches. However, finches may be able to incorporate materials laced with pyrethrin into nests on their own. Similar "self-application" methods have been used successfully used to treat tick infestations in nests of white-footed mice (*Peromyscus leucopus*) (Mather et al., 1987). Preliminary data show that finches had no preference for cotton treated with pyrethrin over cotton treated with water (Koop, unpub. data). Finches were extremely eager to use this material in their nests, taking cotton within seconds of researchers setting it out. Although this approach shows promise, further research is needed to determine whether finches would incorporate sufficient amounts of treated cotton to effectively reduce *P. downsi* abundance in nests.

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Appendix

Additional Calculations

The following calculations to estimate fledging success in infested and uninfested nests during wet and dry years are based on fledging data summarized in Figure 6.4.

Binomial distribution:

$$f(k;n,p) = \binom{n}{k} p^k (1-p)^{n-k}$$

Suppose measurements of treated nests yield c_k c_k nests with k fledglings for $k = 0, 1, 2,\ldots$

Then, the max likelihood estimator for (n, p) is $MLE = \prod_k f(k;n,p)^{c_k}$.

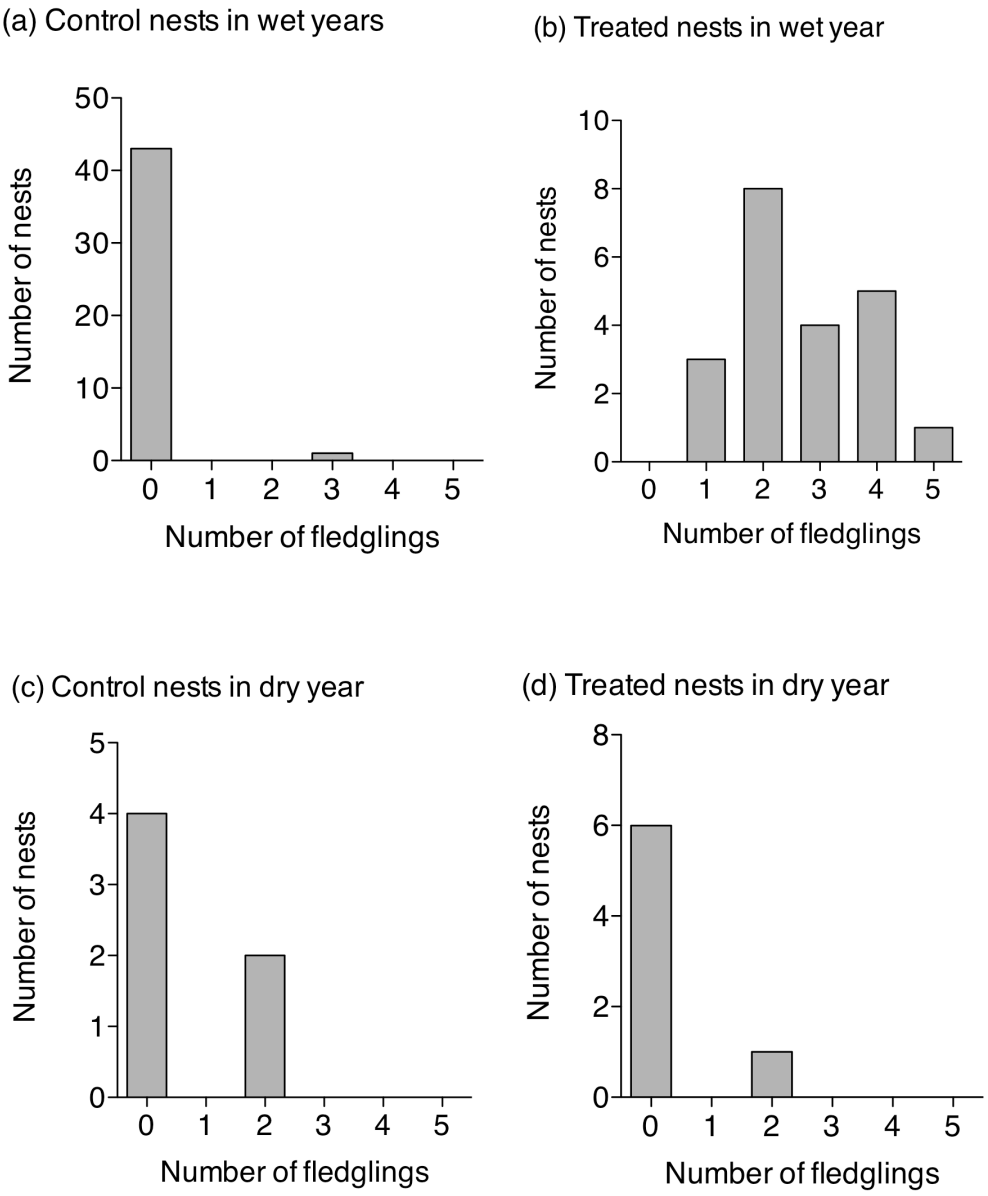

Figure 6.4. Frequency plots of the number of nests to produce a number of fledglings under the following conditions of weather and *P. downsi* parasitism: (a) Control nests during wet years, 2008 and 2010, (b) Treated nests during wet year, 2010, (c) Control nests during dry year, 2009, (d) Treated nests during dry year, 2009. The data shown correspond to the number of fledglings sighted after they left the nest.

So the log likelihood is

$$L = \log(MLE) = \sum_k c_k \log(f(k;n,p))$$
$$= \sum_k c_k \log(\binom{n}{k} p^k (1-p)^{n-k})$$
$$= \sum_k c_k (\log \binom{n}{k} + k \log p + (n-k)\log(1-p))$$
$$= \sum_k c_k \log \binom{n}{k} + \sum_k c_k (k \log p + (n-k)\log(1-p))$$

Now, assume n is fixed and we want to find the p that maximizes L. Then,

$$0 = \frac{dL}{dp} = \sum_k c_k (\frac{k}{p} - \frac{n-k}{1-p})$$
$$= \frac{\sum c_k k}{p} - \frac{\sum c_k (n-k)}{1-p}$$

so,

$$\frac{\sum c_k k}{p} = \frac{\sum c_k (n-k)}{1-p}$$
$$\Rightarrow (1-p)\sum c_k k = p \sum c_k (n-k)$$
$$\Rightarrow \sum c_k k - p \sum c_k k = p \sum c_k$$
$$\Rightarrow \sum c_k k = p \sum c_k k + p \sum c_k (n-k)$$
$$\Rightarrow \sum c_k k = p \sum c_k (k+n-k) = pn \sum c_k$$
$$\Rightarrow p = \frac{\sum c_k k}{n \sum c_k}$$

which means that np is the expected value, based on the measurements. We can then substitute this expression back into the log likelihood L to find which n gives the maximum likelihood.

For infested nests in wet years, we obtain $p = \frac{8}{44n}$ $p = \frac{8}{44n}$, and

$$L = \sum_k c_k \log(\binom{n}{k}) + \sum_k c_k (k \log p + (n-k)\log(1-p))$$

$$= 39\log(\binom{n}{0}) + 3\log(\binom{n}{1}) + \log(\binom{n}{2}) + \log(\binom{n}{3}) + 39n\log(1-p) + 3(\log p + (n-1)\log(1-p)).$$

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CHAPTER 7

CONCLUSION

My dissertation focuses on the effects introduced parasites can have on naïve host populations. Low host specificity and high rates of dispersal have likely facilitated the invasion of *Philornis downsi* into the nests of possibly every land bird species in the Galapagos archipelago (Dudaniec et al., 2008). High *P. downsi* prevalence has been recorded on multiple islands, across variable habitats, and on many diverse avian host species (Wiedenfeld et al., 2007, Dudaniec et al., 2007, O'Connor et al., 2010, Fessl & Tebbich, 2002). The generalist nature of *P. downsi* suggests that all landbird species attempting to breed in the Galapagos will encounter *P. downsi*. In my work, I demonstrate experimentally that high parasite prevalence and virulence, combined with ineffective host defenses, leads to extremely high levels of parasite induced host mortality. If the effects of *P. downsi* on other avian species are similar to those observed for medium ground finches, this fly could be a major threat to avian diversity in the archipelago.

While Darwin's finches are famous for their evolutionary plasticity, human intervention may be necessary, at least in the short-term, to prevent the extinction of these iconic birds due to *P. downsi* parasitism. Conservation efforts and priorities for Darwin's finches may benefit from studies in similar systems. To this end, below I briefly review studies of other avian hosts affected by *P. downsi* or blowflies with similar larval feeding habits.

There are at least sixteen diverse avian host species (across 9 families in 3 orders) known for *P. downsi* in Trinidad, where the fly was originally described (Dodge & Aitken, 1968, Fessl & Tebbich, 2002). The full distribution of *P. downsi* is unknown, though *Philornis* species have been documented throughout Central and South America, and even southern United States (Skidmore, 1985, Couri, 1985). To my knowledge, there are no studies published on the effects of *P. downsi* on the fitness of hosts other than Darwin's finches. Several host species, all Passeriformes, are described from Brazil, but no data are available concerning fitness effects on these host (Fessl et al., 2001). With the exception of one other species, *P. downsi* and *P. falsificus*, all species of *Philornis* larvae live subcutaneously on hosts, or are coprophagous or saprophagous (Dudaniec & Kleindorfer, 2006, Lowenberg-Neto, 2008). *P. falsificus* has a broad geographical range including specimens from Ecuador, Costa Rica, Trinidad and Spain (Leite et al., 2009, Dodge & Aitken, 1968). Like *P. downsi, P. falsificus* is not host-specific, which may provide additional opportunity for future comparative studies on host fitness effects.

Studies of the genus of bird blowflies, *Protocalliphora*, may provide insight where *Philornis*-based studies are lacking. *Protocalliphora* have similar ecology to *P. downsi* although they are rarely cited as the cause of significant nestling mortality (Sabrosky et al., 1989). *Protocalliphora* larvae are free-living and require blood meals from birds to progress from instars to pupae (Bennett & Whitworth, 1991). Larvae are similar in size to *P. downsi* larvae, and are therefore assumed to require similar sized blood meals from hosts. The genus *Protocalliphora* is distributed throughout North America and Mexico as well as much of Europe and Asia. The genus contains generalist and specialist species, similar to *Philornis* (Lowenberg-Neto, 2008); as a genus they are

know to parasitize at least 150 species of birds, from at least 9 orders (Sabrosky et al., 1989). Of these host species, only three are on the International Union for Conservation of Nature (IUCN) "Red List" as near threatened or vulnerable. In all three cases habitat loss is likely the primary cause of population declines, not infestation by *Protocalliphora*.

By comparison, *P. downsi* on the Galapagos are exceptionally virulent and have already been implicated in severe declines of several Darwin's finch species (Fessl et al., 2010, Grant et al., 2005). Unfortunately, the reasons for differences in virulence between these two genera of parasites are unclear. Reported prevalence and intensities of *Protocalliphora* in host nests are extremely variable, fully encompassing the prevalence and intensities found in our own studies of *P. downsi* (Hannam, 2006, Dawson et al., 2005, Gold & Dahlsten, 1983, Morrison & Johnson, 2002). Eastern bluebirds in parasitized nests weighed less at fledging than nestlings in nests where parasites were removed, but there were no significant effects on fledging success (Roby et al., 1992). These birds were affected by an average of 95 *Protocalliphora* per nest, more than double the *P. downsi* intensity observed in our studies. In a similar experimental manipulation, house wren fledging success did not differ significantly between nestlings parasitized by < 15 larvae per nestling, compared to those in unparasitized nests (Johnson & Albrecht, 1993); although postfledging survival may have been compromised by lower haemoglobin levels in parasitized birds (O'Brien et al., 2001). Thus, high parasite intensity does not appear sufficient to fully explain the high levels of mortality observed in our study.

Some studies report increases in nest sanitation behavior and food allocation to parasitized nestlings as a means of reducing the effects of nest fly parasitism (Hurtrez-

Bousses et al., 1998, Hurtrez-Bousses et al., 2000, Ontiveros et al., 2008). While we observed similar behaviors in medium ground finches, the changes were not effective in reducing the harmful effects of *P. downsi* on nestling survival (Chapter 5). An interesting study by Smith (1968), suggested that hosts can build their nests near nonstinging wasp nests as a deterrent to *Philornis*. Over the course of our 3-year study, medium ground finch nests were observed within 1 meter of wasp nests (unidentified species) on only four occasions (out of ~ 160 nests observed). Further work is needed to investigate whether other species of finches or finches in other habitats increase the frequency at which their nests are built near wasp nests.

Bennett and Whitworth (1992) determined that hosts were likely to suffer from higher infestations of *Protocalliphora* when they: re-used nests or nested in the same area from year to year; had nests kept clear of moisture and faeces and built with materials that prevented larvae from falling through the nest; and, were infested by only a single species of *Protocalliphora*. Medium ground finches do not reuse nests, although they do often nest in the same area from year to year (Grant, 1986). Finches build their nests out of tightly woven materials such as grass and sticks. Nest bottom thickness did not correlate with *P. downsi* intensity across multiple species of Darwin's finches, though larger nests were able to support more larvae (Kleindorfer & Dudaniec, 2009). Finally, *P. downsi* is the only known species of botfly to parasitize finches on the Galapagos. Multiple species of *Philornis* have been identified in Trinidad (Dodge & Aitken, 1968). It would therefore be possible to investigate whether interspecies parasite competition occurs with *P. downsi* and whether hosts affected by multiple species of *Philornis* are similarly affected.

Given the similarities between *Philornis* and *Protocalliphora*, methods to control one group of flies could work effectively on the other group. Unfortunately, there are few studies that discuss control methods for populations of *Protocalliphora* (reviewed in Sabrosky et al., 1989). As with our own studies, the use of fumigants, like pyrethrin, were effective in reducing parasite populations in nests affected by *Protocalliphora* larvae. For host species that use nest boxes, the removal of old nest material between breeding seasons reduced parasite loads. However, removal of old material also caused declines in populations of *Protocalliphora* parasitoids. *Nasonia vitripennis*, perhaps the most common parasitoid of *Protocalliphora*, can kill 25-100% of puparia in a nest. Three species of parasitic wasps were found in a shipment of *P. downsi* pupae from the Galapagos (Collignon & Teale, 2010). While there was not evidence that these wasps had hatched from the pupae, two species were identified as chalcid wasps, which are known parasitoids of flies (Sabrosky et al., 1989). Further investigation is needed to determine whether parasitoids of *P. downsi* are already present in the Galapagos, and whether they could be used as an effective biological control agent on *P. downsi* populations.

Darwin's finches live in an extremely variable environment, one in which constant and often rapid adaptation is necessary for survival. The introduction of *P. downsi* to the Galapagos presents the latest challenge to these birds, threatening their very existence. While we can hope that Darwin's finches again become a primary example of evolution by natural selection, imposed by parasites in this case, continued research and conservation efforts are necessary should they be threatened by extinction.

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APPENDIX A

HOW BIRDS COMBAT ECTOPARASITES

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How Birds Combat Ectoparasites

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Abstract: Birds are plagued by an impressive diversity of ectoparasites, ranging from feather-feeding lice, to feather-degrading bacteria. Many of these ectoparasites have severe negative effects on host fitness. It is therefore not surprising that selection on birds has favored a variety of possible adaptations for dealing with ectoparasites. The functional significance of some of these defenses has been well documented. Others have barely been studied, much less tested rigorously. In this article we review the evidence - or lack thereof - for many of the purported mechanisms birds have for dealing with ectoparasites. We concentrate on features of the plumage and its components, as well as anti-parasite behaviors. In some cases, we present original data from our own recent work. We make recommendations for future studies that could improve our understanding of this poorly known aspect of avian biology.

Keywords: Grooming, preening, dusting, sunning, molt, oil, anting, fumigation.

INTRODUCTION

As a class, birds (Aves) are the most thoroughly studied group of organisms on earth. Nevertheless, the adaptive function of many intriguing features of avian morphology, physiology, and behavior are still uncertain. Some of these features are thought to play a role in defense against harmful ectoparasites. Examples include the pectinate middle claw of many birds, the strange odors of some birds, and the odd "maintenance" behaviors, such as sunning, anting or dusting, performed by many birds. In this article we review the ways in which birds are thought to combat ectoparasites. We pay particular attention to possible anti-parasite features of the plumage itself, as well as various forms of anti-parasite behavior. Although the immune system also plays an important role in defense against some ectoparasites, such as blood-feeding mites [1], we do not cover immune defenses in this review. Instead, we refer readers to other papers in this volume, and recent reviews of immunology published elsewhere, e.g. [2] and [3].

In the classic work *Fleas, Flukes and Cuckoos*, Rothschild and Clay [4] catalogued the incredibly rich diversity of parasitic organisms inhabiting birds, including groups as different as viruses, fungi, bacteria, protozoa, worms and arthropods. The major groups with *ectoparasitic* forms are as follows:

1) Insects: Four orders, including lice (Phthiraptera), fleas (Siphonaptera), true bugs (Hemiptera), and flies (Diptera) [5].
2) Mites and ticks (Acari): *many* families [6-9].
3) Leeches: four families [10].
4) Fungi: keratinophilic and cellulose decomposing forms [11].
5) Bacteria: several unrelated groups that decompose feathers [12].

Relatively little was known about the impact of ectoparasites on non-game wild birds until about 25 years ago, when ornithologists began to take a strong interest in parasites. One catalyst was Hamilton and Zuk's influential 1982 [13] paper arguing that the elaborate visual and acoustic displays of many birds evolved as a result of parasite-mediated sexual selection. Since then, dozens of papers testing the impact of parasites on wild birds have been published. For reviews, including the topic of sexual selection, which we will not cover here, see [3, 14-21].

These studies confirm that many ectoparasites are potent agents of selection on birds, affecting both the survival and reproductive components of avian fitness. Not surprisingly, therefore, birds appear to have evolved a wide variety of defenses for controlling ectoparasites. Moyer and Clayton [22] provided a succinct review of defenses involving plumage as a barrier, and antiparasite behaviors of birds. Since their review, several dozen new papers have been published with information pertinent to these kinds of defenses. We review these papers below, and in some cases we report original data relevant to purported defenses.

We consider ectoparasites to include taxa that spend at least some of their life cycle in close association with the host, as opposed to more ephemeral "parasites", such as mosquitoes. We do not cover defenses aimed primarily at

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these ephemeral species, such as fly repelling behavior, defensive sleeping postures, microhabitat choice, territoriality, and "selfish herd" effects. For reviews of these topics see Lehane [23], Hart [24] and Weldon and Carroll [25].

We use parasite *load* in reference to any of the following more precise measures: *richness* (the number of species of parasites present); *prevalence* (the fraction of parasitized individuals in a host population); *intensity* (the number of individual parasites in an infested host); *abundance* (the number of individual parasites in a host, regardless of infestation). Hence, *mean intensity* is the average number of individual parasites across infested hosts in a population, and *mean abundance* is the average number of parasites across all host individuals, regardless of infestation. For further details see Bush *et al.* [26].

PLUMAGE AS A BARRIER

Most ectoparasites are in contact with the plumage, at least some of the time. Some ectoparasites, such as feather lice (Phthiraptera: suborder Ischnocera), are in contact with the plumage *all* of the time. Indeed, they even feed on feathers, which are digested with the aid of endosymbiotic bacteria [27]. It is therefore reasonable to expect that some chemical or mechanical features of the plumage may have evolved to deter ectoparasites, similar to the many features of foliage known to deter herbivorous insects [28]. Plumage related defenses might include feather molt, analogous to the abscission of plant leafs reducing infestations of leaf miners and other endophytic and sessile herbivorous insects (reviewed in Stiling *et al.* [29]).

Feather Molt

Conventional wisdom has it that feather molt helps reduce ectoparasite loads [5]. Indeed, molt presumably does help birds jettison immobile parasites, such as fungi and bacteria that live in the plumage. Burtt and Ichida [30] showed that the abundance of feather-degrading bacteria fluctuates seasonally, with the smallest infestations in the autumn, which is consistent with this hypothesis. But it remains unclear whether molt plays an important role in controlling more mobile parasites, such as mites and lice.

Records of lice on molted feathers suggest that molt may indeed reduce arthropod ectoparasite loads [31]. Post and Enders [32] attributed the low prevalence of lice on Sharp-tailed Sparrows (*Ammodramus caudacutus*), compared to Seaside Sparrows (*A. maritimus*), to the fact that the former molt twice a year, while the latter molt once a year. Several researchers have carried out longitudinal studies in which they documented an apparent reduction in ectoparasite load over the course of the host's molting period [33]. Baum [34] reported an 85% drop in the abundance of lice on molting Eurasian Blackbirds (*Turdus merula*). Markov [35] observed a decrease in the number of ectoparasites on European Starlings (*Sturnus vulgaris*) during the autumn, and argued that feather molt caused this decrease. However, Boyd [36] suggested that seasonal changes in climate were actually responsible for the autumn reductions. Changes in climatic factors - particularly ambient humidity - are known to have a significant impact on ectoparasite abundance, at least in the case of lice [22, 37, 38].

A recent longitudinal study of ectoparasite loads on House Finches (*Carpodacus mexicanus*) indicates that the relationship between molt and ectoparasite abundance can be complicated [39]. The results of this study show that the abundance of two species of feather mites (*Strelkoviacarus* sp. and *Dermoglyphus* sp.) increased, rather than decreased, during the molting season. The louse *Menecanthus alaudae* also increased during the molting season on male House Finches. The authors argued that the energetic cost of molt reduced the amount of energy birds could expend on activities such as preening, leading to an increase in ectoparasites. The authors also compared the ectoparasite loads of birds in various degrees of molt. Molting males had more feather mites than non-molting males, whereas the number of mites on molting vs. non-molting females did not differ significantly. In addition, the study showed that molting males had more lice than molting females. The authors suggest that these patterns are driven by the additional energetic costs associated with the possession of showy plumage in males.

Moyer *et al.* [40] conducted an experimental test of the impact of molt on ectoparasites. The authors manipulated photoperiod to trigger early molt in captive Rock Pigeons (*Columba livia*) infested with lice. They then tracked the abundance of lice on molting and non-molting (control) birds over the course of several weeks. Visual examination of lice on different body regions indicated that feather molt reduced louse abundance. However, body washing, a more robust method of quantifying lice [41], showed that molt did not, in fact, reduce the abundance of lice. Two factors caused visual examination to underestimate the number of lice on the molting birds. First, molt replaced worn feathers with new, lush plumage that obscured lice during visual examination. Second, lice sought refuge inside the sheaths of newly developing feathers, where they could not be seen. The illusion of reduced louse abundance documented by Moyer *et al.* [40] calls into question observational studies documenting apparent reductions in lice during molt. This may also be true for other ectoparasites.

A few studies of molt have used methods for quantifying ectoparasites that are more rigorous than visual examination. For example, Chandra *et al.* [42] fumigated Common Mynas (*Acridotheres tristis*), ruffled their plumage, and quantified the lice. McGroarty and Dobson [43] used body washing to determine the number of lice on House Sparrows *(Passer domesticus)*. Both studies showed a reduction in louse abundance in late summer, coincident with the postnuptial molt of the host. However, experimental manipulations are still needed to establish molt as the cause of these decreases, rather than some third factor that covaries with both molt and ectoparasite reductions. One such factor could simply be transmission of lice from parent to offspring birds. Lice typically move in large numbers from parent birds to their offspring at the end of the breeding season, leading to a decrease in the abundance of lice on adult birds around the time of molt [44-47]. Dispersal of lice to juveniles could also explain why fewer newly deposited feather louse eggs are found on adult feathers near the end of the host's breeding season [48].

Host physiological constraints may give many ectoparasites time to circumvent molt, which tends to be a gradual process in most birds because thermal insulation and aerody-

namic efficiency are both compromised in proportion to the number of missing feathers [49, 50]. Energy is also required, of course, to create each new feather [51]. Feather quality can be inversely proportional to the rate of molt, further suggesting constraints on rapid molt [52]. If feathers are lost gradually, then it may be possible for ectoparasites to avoid feathers that will soon be molted. A survey of feather mite distributions on the flight feathers of molting passerines shows that mites can, in fact, avoid molting feathers [53]. Similarly, mites that live inside the quills of feathers (Syringophilidae) are known to abandon the old feathers in favor of new ones before the old ones molt [54, 55].

Two mechanisms have been proposed to explain how ectoparasites detect and avoid molting feathers [56]. The "vibration" hypothesis proposes that the cue used by the ectoparasites is the vibration caused by rocking of the old feather as it is pushed out of a follicle by the newly emerging feather. The "window" hypothesis proposes that ectoparasites on sequentially molting flight feathers can detect changes in movement or airflow caused by absence of the adjacent, molted feather. Pap *et al.* [57] addressed both hypotheses in a clever experiment. To test the window hypothesis they removed the sixth primary feather from non-molting Barn Swallows *(Hirundo rustica)*. To test the vibration hypothesis they cut part way through the shaft of the sixth primary feather of the opposite wing to simulate vibration in a molting feather. The simulated window did not cause mites to leave the adjacent (seventh) primary on the first wing, suggesting that the vibration hypothesis might be the correct explanation. Unfortunately, the authors did not report whether mites left the partially cut sixth primary on the opposite wing, nor did they report the number of mites on the fifth primary, which is one place the mites would be expected to move in response to vibration. The authors did note a decrease in the number of mites on the eighth primary, but the relevance of this observation is unclear. Interestingly, experimental birds with pulled or cut feathers had significantly fewer mites on the flight feathers than did control birds at the end of the experiment. The authors suggested that the mites may have moved from flight feathers onto body feathers to escape molt, but there were no data with which to test this hypothesis.

Jovani *et al.* [56] also evaluated the two hypotheses using mites on Barn Swallows. As in many birds, there is a time lag between molting of a primary feather, which creates a window, and hypothesized vibration in the adjacent feather before that feather also molts. Jovani *et al.* [56] found that mites stayed on feathers near the window for a long time, moving only when the feather was nearly ready to drop. As in the case of Pap *et al.*'s study [57] this observation suggests that vibration may be a more important cue than the appearance of a window. However, additional experimental manipulations of cues that ectoparasites could use to detect molt are needed for a more complete understanding of this question.

Feather Toughness

Feathers containing melanin—the pigment typically responsible for brown, gray or black colors [58]—are more resistant to mechanical abrasion than feathers without melanin [59, 60]. This toughness makes melanin rich feathers more resistant to wear and tear, and may also deter feather-feeding ectoparasites. Two studies suggest that melanin can limit damage by feather feeding lice [61, 62]. Kose and colleagues [61] surveyed feather damage in Barn Swallow (*Hirundo rustica*) populations and found that the holes chewed by lice were significantly more likely to occur in the white (melanin-free) spots on the tail feathers, compared to black (melanin-rich) regions of the tail. The authors conducted a louse-preference trial *in vitro* and found that the lice preferred to be on white portions of the tail feathers. Unfortunately, recent evidence indicates that the louse genus studied by Kose *et al.* [61] is not the one that creates holes in the feathers, thus bringing into question the relevance of their experiment. Kose *et al.* [61] studied preferences of the louse *Machaerilaemus malleus* (synonym: *Hirundoecus malleus*), in the family Menoponidae, whose members often feed on blood and feathers [63]. The holes in Barn Swallow tail feathers appear to be caused by members of the genus *Brueelia* [64], in the family Philopteridae, whose members typically feed on feathers and dead skin. The experiments performed by Kose *et al.* [61] need to be repeated using *Brueelia*.

Experiments conducted by Bush *et al.* [65] indicate that melanin does not have an effect on feather-feeding lice from Rock Pigeons. The authors captured pigeons of different color morphs ranging from white to black. Feather-feeding lice (*Columbicola columbae,* and *Campanulotes compar*) were fed feathers from these birds *in vitro*. After two weeks, there was no significant difference in the amount of feather material consumed, nor in the survival of lice on feathers with different amounts of melanin. Additional experiments with *C. columbae* showed that there was no significant difference in reproduction of lice on white vs. black feathers, nor did the lice exhibit a preference for different colored feathers.

Melanized feathers may be more resistant to feather-degrading bacteria (FDB). Three studies have addressed this question by exposing *Bacillus licheniformis*, a common strain of FDB, to melanized and unmelanized feathers. Goldstein *et al.* [66] suggested that melanized feathers resisted degradation by FDB; however, this study was performed without adequate controls or replicates [67]. In contrast, Grande *et al.* [68] found that FDB actually degraded melanized feathers faster than unmelanized feathers; however, in this study feather degradation was scored visually, which may be problematic because color could bias human perception of degradation. In an attempt to remedy these shortcomings, Gunderson *et al.* [67] conducted an experiment where goose feathers were inoculated with *B. licheniformis*. They found that melanized feathers had lower bacterial densities, degraded more slowly, and had less degradation than unmelanized feathers, indicating that melanin does, in fact, deter at least one strain of FDB *in vitro*.

Many species of birds have melanic morphs, and the darker morphs typically live in more humid regions – a pattern known as *Gloger's rule* [69]. Burtt and Ichida [70] hypothesized that this pattern may be driven by FDB, which thrive in humid conditions. They compared the degradation rates of *B. licheniformis* isolated from darkly colored Song Sparrows (*Melospiza melodia*) from a humid region and more lightly colored Song Sparrows from an arid region. By growing

these bacterial isolates on chicken feathers under "common garden" laboratory conditions, the authors showed that the bacteria from the humid region degraded feathers faster than bacteria from the arid region. Burtt and Ichida [70] suggested that Song Sparrows in humid regions (where bacteria do better) evolved more melanin because of increased pressure from the more detrimental strain of *B. licheniformis*.

In another study, Cristol *et al.* [71] inoculated the feathers of live birds with *B. licheniformis* to test the impact of sunning behavior on FDB. They noticed that darkly colored European Starlings (*Sturnus vulgaris*) had far less damage than more lightly colored Northern Cardinals (*Cardinalis cardinalis*). However, as the authors themselves point out, these data are only suggestive, since the experiments with starlings and cardinals were run at different temperatures, humidities, and for different lengths of time.

In summary, studies with *B. licheniformis* [70, 71] suggest that melanin may be an important defense against FDB. However, experiments manipulating bacteria *in vivo* on light and dark birds are needed for a more convincing test of this hypothesis, as well as to test the fitness consequences of FDB for birds [72]. Moreover, studies are needed to understand how melanin affects bacterial communities. Interactions between bacteria could alter how we interpret the role of melanin as a bacterial defense. Experiments done *in vivo* should, if possible, incorporate whole communities of FDB, not just *B. licheniformis,* which is often studied because it can be cultured *in vitro*. Work is also needed to elucidate the precise mechanism(s) by which melanins deter bacteria [67]. It is entirely possible that an antibacterial role of melanin could have more to do with its influence on the avian immune system [3] than it's influence on feather hardness (see other articles in this volume).

FEATHER TOXINS

Toxins in the plumage of some birds may help combat ectoparasites [25, 73]. The best-known example is batrachotoxins in the feathers and skin of several species in the New Guinea passerine genera *Pitohui* and *Ifrita* [74, 75]. Batrachotoxins, which are also found in the skin of poison dart frogs, (*Phyllobates spp.*) are thought to play a role in deterring predators. Experimental evidence suggests that the toxins also deter ectoparasites [76-78]. Dumbacher [78] conducted a series of *in vitro* trials in which he exposed feather lice from a variety of bird species to feathers of *Pitohui* and other non-toxic birds. He found that lice avoid feeding or resting on *Pitohui* feathers when they are given a choice. Lice on *Pitohui* feathers also show higher mortality than lice on non-toxic feathers. Since batrachotoxin detrimentally affects a wide variety of invertebrates [78], it may deter other ectoparasites in addition to lice. Interestingly, a survey of 30 New Guinea passerine genera showed that *Pitohuis* had the lowest tick loads [79]. Another study showed that the family Pachycephalidae, which includes the genus *Pitohui,* has comparatively few arthropod-vectored haematozoan parasites [80].

Odorous Feathers

At least 80 genera of birds in 17 orders produce odors that humans can readily detect [81]. It is possible that one adaptive function of such odors is to combat ectoparasites. This hypothesis has been tested most thoroughly in Crested Auklets (*Aethia cristatella*) (Fig. **1**), which emit a pungent citrus-like odor that humans can detect at a considerable distance from breeding colonies [82]. Douglas *et al.* [83] identified the odor constituents as a series of short-chained, saturated and monounsaturated aldehydes, which are corrosive irritants that are volatile and reactive. The authors suggested that the citrus odor might repel ectoparasites since two of the major constituents, hexanal and octanal, are known arthropod repellents.

Douglas *et al.* [84] tested the effect of synthetic versions of auklet odorant compounds on two genera of auklet lice (*Austomenopon* sp. and *Quadraceps* sp.). Lice exposed to 1µl of either octanal or Z-4-decanal became moribund in seconds. In contrast, when Douglas *et al.* [85] exposed Rock Pigeon lice (*Columbicola columbae* and *Campanulotes compar*) to fresh auklet feathers placed in covered petri dishes with lice, or to fresh auklet carcasses sealed in beakers with lice, there was no effect on parasite survival. Douglas *et al.* [85] also compared the relative abundance of lice on Crested Auklets to lice on Least Auklets *(A. pusilla)*, which do not emit a noticeable odor (the birds were from the same mixed breeding colony). They found that Crested Auklets actually had significantly more lice than Least Auklets, even after controlling for a difference in host body size.

Douglas *et al.* [84] also tested the effect of synthetic versions of auklet odorant compounds on two species of ticks. Laboratory reared ticks (*Amblyomma americanum*) were exposed to octanal on an artificial host consisting of filter paper attached to a heated, rotating drum. Ticks detached

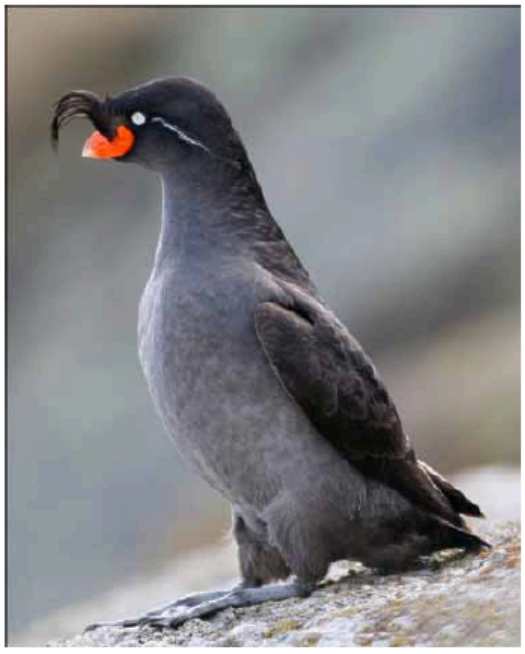

Fig. (1). Crested Auklets (*Aethia cristatella*), such as the one shown here, emit a citrus-like odor that may deter ectoparasites. Photo by S. Gross.

significantly faster from artificial hosts treated with 10% octanal than from artificial hosts treated with ethanol. Ticks were also exposed to a synthetic cocktail designed to mimic the diverse chemical composition of auklet odorant. Ticks challenged with this cocktail (40% octanal, 21% hexanal, 8% *Z*-4 decenal, 3% decanal, 7% hexanoic acid, and 3% octanoic acid) showed a dose dependent response. Ticks exposed to at least a 10% dilution of the cocktail remained attached to the artificial host for a shorter period of time than controls. Douglas *et al.* [84] also conducted experiments with *Ixodes uriae*, the tick found on Crested Auklets in nature. The results were similar to the experiments conducted with *A. americanum*. Moreover, when these ticks were placed in a vial with 5µl octanal, they became moribund within an hour.

In another study involving *I. uriae* ticks, Douglas [86] quantified the relative odor emissions from 57 live Crested Auklets. Interestingly, the individual with the lowest emission level was infested with 14 ticks. Only one other bird was infested (with two ticks), out of 96 birds surveyed in the same breeding colony. In contrast, Hagelin [87] found no evidence that *I. uriae* ticks are repelled by fresh Crested Auklet feathers placed in petri dishes, compared to feathers of Least Auklets or Parakeet Auklets *(A. psittacula),* neither of which emit a noticeable odor.

Hagelin and Jones [81] have argued that the repellency studies conducted by Douglas *et al.* [84] used synthetic compounds that exceed natural concentrations (c.f. those measured in auklet odorant by Hagelin *et al* [88]). In response, Douglas [89] argued that Hagelin *et al.*'s study underestimated the quantity of volatiles in auklet feathers because the samples were kept under suboptimal conditions, during which time they may have degraded (and see Hagelin [90]).

Douglas [91] published data indicating that natural concentrations of auklet odor are, at least in some cases, greater than those published by Hagelin *et al* [88]. He also conducted *in vitro* experiments with ticks (*A. americanum*) exposed to low doses (0.5% and 1%) of a synthetic cocktail. Douglas [91] argued that these doses simulate natural conditions because the 1% solution exposed ticks to lower concentrations of octanal than he isolated from the crown and nape feathers of Crested Auklets. Locomotion of ticks at both doses in this study was significantly less than that of controls, and there was evidence of paralysis in some of the ticks exposed to the 1% treatment. These results suggest that the compounds in Crested Auklet odorant do have the potential to deter ticks. What is needed for a more definitive study, if possible, is a test of the impact of the odorant on ticks under natural conditions in the field. Ideally, this test would involve some kind of experimental manipulation of odorant levels. Tests for an impact of odorants on ectoparasites in other groups of birds are also needed.

Uropygial Oil

Most birds have a nipple-like uropygial (preen) gland on their rump. They squeeze this protuberance with their bill during preening and spread its oil throughout the plumage. The oil is known to help maintain plumage strength and flexibility, but it has long been thought that the oil may also deter ectoparasites [77, 92, 93]. Uropygial oil could combat ectoparasites by reducing their mobility on feathers or skin. If the oil coats the exterior of a parasite, or at least plugs the spiracles (breathing holes) of arthropod parasites, it might also suffocate them [94]. In some species of birds the oil is associated with noxious or repellent odors, which could conceivably affect ectoparasites [73].

Moyer *et al.* [94] tested whether preen oil helps Rock Pigeons combat feather lice. They compared the survival of lice raised in an incubator on feathers treated with uropygial oil to the survival of lice on control feathers without oil. They found that lice on oiled feathers died more rapidly than controls. They also compared the population dynamics of lice on captive pigeons with intact uropygial glands to lice on pigeons with their glands surgically removed. Removal of the gland had no significant effect on louse populations over a period of four months (about 5 louse generations). This finding suggests that birds do not "fumigate" themselves with preen oil, despite the fact that the oil does, in fact, have the capacity to kill lice when applied *in vitro* [94].

Uropygial oil may inhibit the growth of certain pathogenic bacteria and fungi that inhabit the plumage of birds [11,95-99]. Jacob *et al.* [99] demonstrated that constituents of Pelecaniform uropygial oil, applied *in vitro*, have a dose-dependent inhibitory effect on Gram-positive bacteria and fungal dermatophytes (*Trichophyton* sp., *Microsporum gypseum*). The Red-billed Woodhoopoe (*Phoeniculus purpureus*), like other species of woodhoopoes, emits a malodorous secretion from its uropygial gland [73]. Law-Brown [98] identified 17 chemical constituents found in the uropygial oil of this species. Using disc-diffusion assays, she tested the *in vitro* activity of each constituent against 13 pathogenic bacterial strains (e.g., *Salmonella enteritidis, Staphylococcus aureus,* and *Streptococcus faecalis*), and against a strain of the feather-degrading bacterium *Bacillus licheniformis* [30]. Seven of the constituents significantly inhibited bacteria, suggesting that uropygial oil has the potential to combat bacterial infections and concommitant feather degradation.

Interestingly, most of the chemical constituents of the uropygial oil of Red-billed Woodhoopes are synthesized by yet another bacterium, *Enterococcus phoeniculicola*, which lives in the bird's uropygial gland. Law-Brown [98] treated the glands of this species with an antibiotic, and then compared the chemical composition of their uropygial oil to that of untreated controls. Her results showed that only two of 17 constituents were still present following antibiotic treatment. Furthermore, the ones that remained in the oil (e.g., cholesterol) were present at elevated levels, suggesting they were no longer metabolized in the absence of the bacteria. This pioneering study is the first to document a bacterial symbiont that metabolizes constituents of uropygial oil.

Uropygial oil affects different strains of parasitic bacteria and fungi in different ways. Pugh and Evans [96] tested the impact of European Starling (*Sturnus vulgaris*) "feather fats" on four species of keratinophilic fungi. They made the interesting observation that, while sporulation of *Chrysosporium keratinophilum* increased, the same oils inhibited the growth of *Arthroderma quadrifidum, A. uncinatum* and *Ctenomyces serratus*. Pugh [11] found that the feather fats of Blackbirds (*Turdus merula*) inhibited the growth of *C. serratus*, while stimulating the growth of *A. curreyi*. In a similar study, Bandyopadhyay and Bhattacharyya [100] tested the effects of uropygial oil on several fungal species cultured from the skin

of white leghorn fowl. They found that surgical removal of the uropygial gland led to an increase in the populations of all but one species of fungi.

Shawkey *et al.* [101] suggest that uropygial oil might benefit birds by promoting the growth of bacteria or fungi that out compete or otherwise exclude more virulent microbes. The authors identified 13 bacterial isolates from the feathers of wild house finches (*Carpodacus mexicanus*), and measured the feather-degrading activity of each. They tested the effects of uropygial oil on the survival and growth of each strain through a disc-diffusion assay. They found that uropygial oil inhibited the growth of three feather degrading strains, including *Bacillus licheniformis*, but it had less of an effect on more benign strains. Future studies should aim to clarify the impact of uropygial oil on bacterial and fungal strains both in isolation, and in the context of the full microbial community.

Feather mites may have an entirely different relationship with uropygial oil. Blanco *et al.* [102] suggested that feather mites are commensals, or even mutualists, rather than parasites. The main food resource of certain feather mites is uropygial oil on the feathers. Along with the oil, the mites consume microbes such as fungi and bacteria [9]. If these microbes include forms that are dangerous to the bird, the consumption of uropygial oil by feather mites may be beneficial to the host [103, 104]. This interesting hypothesis should be tested experimentally.

BODY MAINTENANCE BEHAVIOR

Grooming behavior, defined as preening and scratching combined [105], is known to be critical for defense against ectoparasites [5, 24]. Preening is of two types: self-preening (Fig. **2a**) and allopreening (Fig. **2b**). Water bathing, dusting, sunning, anointing and cosmetic behaviors may also play a role in ectoparasite defense. Below we review the evidence relevant to each of these behaviors, as well as the evidence relevant to the different types of grooming.

Grooming: Self-Preening

Preening is the most common defensive behavior that birds use against ectoparasites. Preening involves the bird pulling its feathers between the two mandibles of the bill, or nibbling the feathers with the bill tips. Birds can spend a significant portion of their daily time budget preening; e.g. Losito *et al.* [106] showed that juvenile mourning doves spend up to 23% of their time preening. This is a considerable amount of time and energy, given that the cost of preening can be about twice the basic metabolic rate [107]. Croll and McLaren [108] documented a nearly 200% increase in the metabolic rate of preening Thick-billed Murres (*Uria lomvia*), compared to resting individuals. The increase was higher than that associated with either feeding (49%) or diving (140%).

Many studies have shown that preening is a critical defense against ectoparasites. The defensive role of preening was initially suggested by natural "experiments" in which birds with bill deformities have very high ectoparasite loads [4, 5, 31, 34, 36, 109-113]. For example, Clayton *et al.* [113] observed that among 150 wild Rock Pigeons, the three individuals with the most feather lice all had minor bill deformities. One of the deformed individuals had more than 10,000 lice, compared to a mean of 631 lice on birds without deformities. Of course, birds with deformed mandibles may have other problems, such as impaired foraging ability, which could contribute to increases in ectoparasite load [114-117]. Therefore, a rigorous test of the role of preening in ectoparasite control requires an experimental approach that alters only preening efficiency.

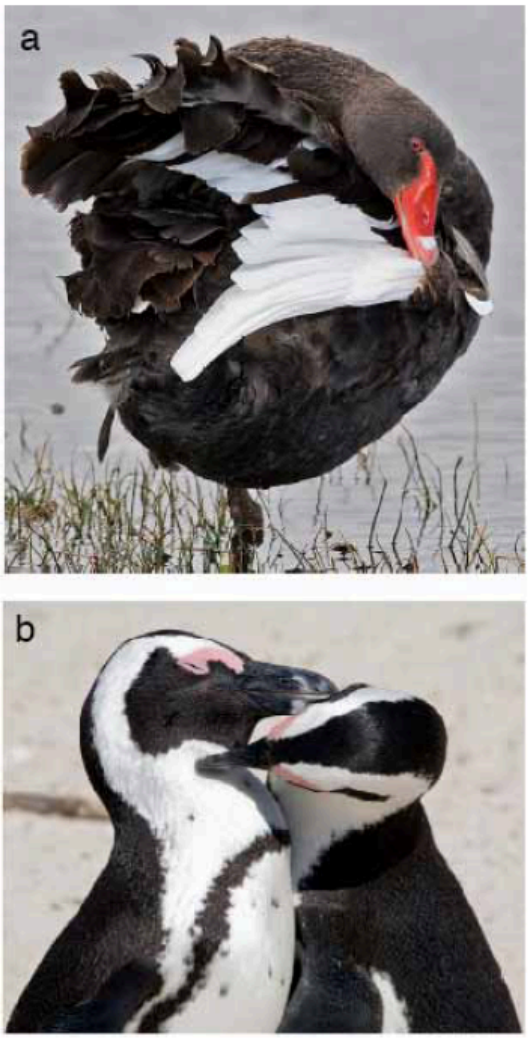

Fig. (2). (**a**) Preening Black Swan (*Cygnus atratus*). Photo by Noodle Snacks (commons.wikimedia.org). (**b**) Allopreening between Magellanic Penguins (*Spheniscus magellanicus*). Photo by Andreas Edelmann (fotolia.com).

Early such tests impaired preening crudely by clipping ca. 1 cm from the upper mandible of domestic chickens or pigeons, leading to dramatic increases in ectoparasite load [118-121]. Subsequent tests impaired preening in a less invasive way, using poultry "bits," which are small, C-shaped

pieces of metal or plastic. Bits are inserted between the upper and lower mandibles and crimped slightly in the nostrils to prevent dislodging, but without damaging the tissue. They create a 1-3 mm gap between the mandibles that impairs the forceps-like action of the bill required for efficient preening. Bitting triggers a dramatic increase in feather louse populations on pigeons [112, 113, 122, 123]. This increase is not due to side effects of bits, such as an impact on feeding, because pigeons feed on whole grain (corn, peas, etc.) that can be picked up despite the small mandibular gap created by the bits. Clayton and Tompkins [123] showed that bits have no effect on the survival or reproductive success of (unparasitized) Rock Pigeons, compared to non-bitted controls.

The importance of preening for ectoparasite control is also apparent from comparative studies. The size of the bill overhang varies markedly across species of birds (Fig. **3**). For example, Clayton and Walther [124] compared the diversity of lice among 52 species of Peruvian birds belonging to 13 families. Phylogenetically independent comparisons revealed a significant negative correlation between louse abundance and degree to which the upper mandible (maxilla) overhangs the lower mandible. This correlation suggests that birds with slightly longer overhangs are better at controlling lice by preening. Extreme overhangs, such as the hooked bills of raptors and parrots, are adaptations for feeding that do not enhance preening efficiency [124].

Clayton *et al.* [125] demonstrated how the maxillary overhang functions to control lice. Experimental removal of the tiny (1-2 mm) overhang (Fig. **3e,f**), triggered a dramatic increase in louse population size (Fig. **4a**). Regrowth caused the louse populations to subsequently crash (Fig. **4a**). In a series of measurements using magnetic transducers glued to the mandibles of birds, the authors showed that the lower mandible moves forward during preening (Rock Pigeon preening at 1/4th actual speed, Rock Pigeon preening at 1/24th actual speed) (suppl 1). This forward motion, which was remarkably fast, at up to 31 times per second, created a shearing force against the overhang that damaged the lice (Fig. **4b-e**). Without the maxillary overhang, birds were unable to generate this force. Additional experiments showed that removal of the overhang had no impact on feeding efficiency, suggesting that the overhang is a specific adaptation for ectoparasite control. Overhangs longer than a mean of 1.5mm broke significantly more often than shorter overhangs, further suggesting that stabilizing selection favors overhangs of intermediate length. Considering the critical

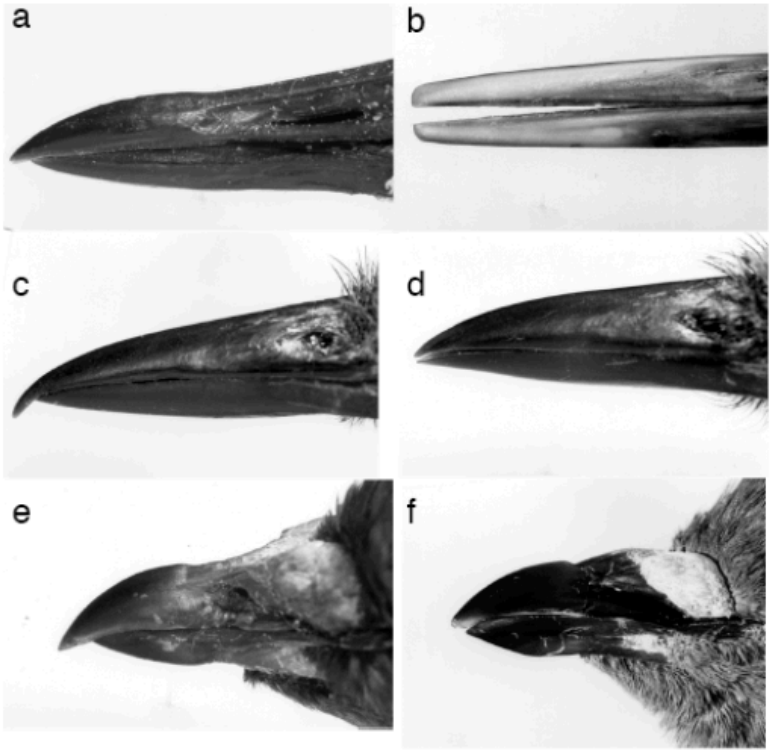

Fig. (3). Natural and experimentally induced variation in the bill overhang. Within the family Charadriidae, the Black-bellied Plover (*Pluvialis squatarola*) has a pronounced overhang (**a**), whereas the Black Oystercatcher (*Haematopus bachmani*) lacks an overhang (**b**). Within the species Western Scrub-Jay (*Aphelocoma californica*), populations living in scrub oak have a pronounced overhang (**c**), whereas those living in pinyon pine have no overhang (**d**). Rock Pigeons (*Columba livia*) have a pronounced overhang (**e**), which Clayton *et al.* [125] trimmed using a Dremel® rotary tool (**f**). Trimming is a harmless procedure, and the overhang regrows in 1-2 weeks. The results of trimming are shown in Fig. (**4**). Photos by C. Beittel.

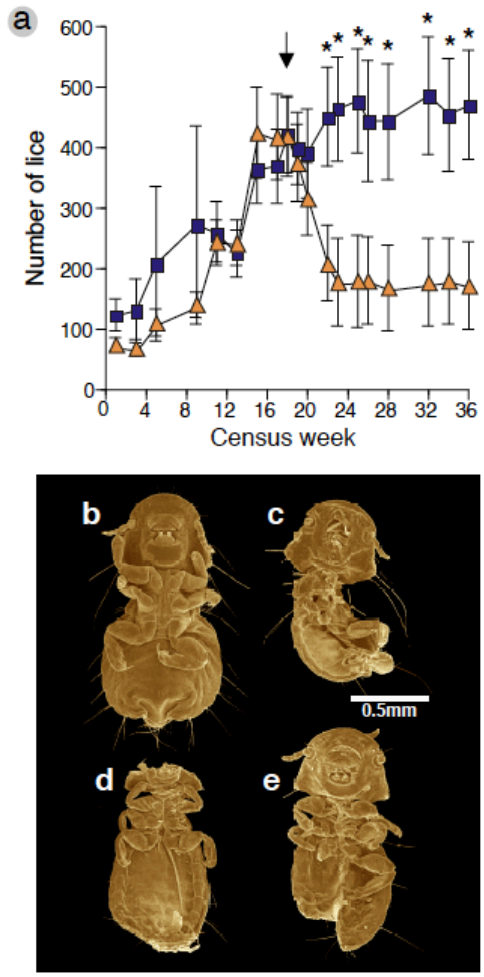

Fig. (4). (**a**) Mean (± 1 SE) number of lice on 26 adult pigeons in an experiment to test the impact of the bill overhang on preening efficiency. The overhangs of all birds were trimmed for 17 weeks; at week 18 (arrow), half the birds (orange triangles) were allowed to regrow their overhangs, while the remaining half (blue squares) continued to be trimmed weekly. Data were analyzed using a 2 (treatment: trim, regrow) X 11 (post-treatment census) ANOVA with repeated measures on the second factor (census). There were significant overall effects of treatment ($P = 0.003$) and census ($P < 0.0001$), and a significant interaction ($P < 0.0001$). Birds allowed to regrow their overhangs had significantly fewer lice than trimmed birds at each of the final eight censuses = weeks 22-36 (Protected $t > 1.97$, df = 240, $*P < 0.001$). (**b**) SEM of an undamaged louse (*Campanulotes compar*), compared to lice that have had most of their legs removed (**c**), or been decapitated (**d**), or lacerated (**e**) by birds with normal overhangs. Reprinted from Clayton *et al.* [125].

importance of the maxillary overhang for controlling lice, Clayton *et al.* [125] concluded that the adaptive radiation of beak morphology in birds should be re-assessed with both feeding and preening in mind.

Interestingly, a negative correlation between length of the bill overhang and ectoparasite abundance is also apparent among populations within species. Populations of the Western Scrub-jay (*Aphelocoma californica*) have bills specialized for feeding in their respective habitats [126, 127]. Scrub-jays in oak habitat have hooked bills (Fig. **3c**), whereas the bills of populations in pinyon habitat are pointed (Fig. **3d**). Moyer *et al.* [128] quantified lice on 170 freshly collected jays and found a significant relationship between bill morphology and louse load. Although louse prevalence was low, infested birds with pointed bills had significantly more lice than infested birds with hooked bills. More recent work using better methods of quantifying parasites further suggests that lice also exert stabilizing selection on the bill morphology of jays (Fig. **5**).

Grooming: Allopreening

In addition to preening themselves, birds sometimes "allopreen" one another (Fig. **2b**). Allopreening helps reduce ectoparasites on the head and neck, which are impossible to self-preen. Allopreening is a widespread behavior observed in many species of birds [129]. It is most common between courting and mated individuals, and between parents and their offspring. Harrison [129] argued that allopreening serves mainly a social function, such as reinforcement of the pair bond, and is of little or no importance for ectoparasite control. However, subsequent studies indicate a role for allopreening in parasite control [130-133]. Radford and Du Plessis [134] suggested a dual function for allopreening in the Green Woodhoopoe (*Phoeniculus purpureus*). Allopreening of the head and neck regions occurs at similar rates for dominant and subordinate individuals, suggesting a hygienic function. However, allopreening of self-accessible body regions, such as the wings, back or breast, are influenced by group size and dominance status, suggesting a social function.

Among the most convincing demonstrations of the importance of allopreening for controlling parasites is Brooke's [131] study of tick-infested Macaroni Penguins *(Eudyptes chrysolophus)*. Brooke reported that individual birds, which could only self-preen, had two to three times more ticks than paired birds, which engaged in frequent allopreening. The ticks were found mainly on the head and neck, suggesting that the larger numbers on unpaired birds were due to the lack of allopreening, rather than inefficient self-preening. It is important to keep in mind, however, that the author could not control for possible covariates of tick load, such as genetic resistance. Such resistance, if present, might have contributed to the low tick loads of some individuals, as well as to their ability to attract mates. Hence, inability to attract mates could lead to a spurious inverse correlation between tick load and allopreening. A more rigorous test of the role of allopreening requires analysis of covariation between allopreening and parasite load [cf. 135] or - even better - experimental manipulation of allopreening and its impact on ectoparasites.

Grooming: Scratching

Scratching with the feet is an important means of controlling ectoparasites on regions that cannot be self-preened, such as the head. Birds with a deformed or missing foot of-

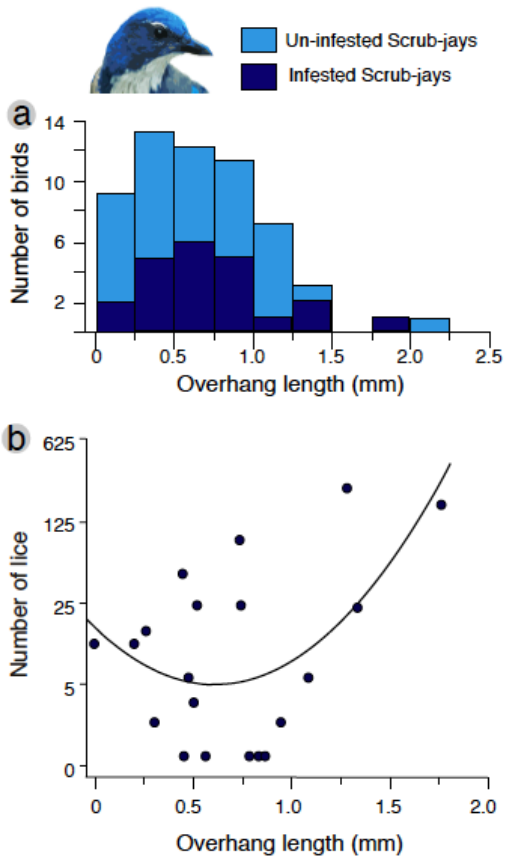

Fig. (5). Overhang lengths of Western Scrub-jays, in relation to ectoparasite abundance. Jay specimens (n = 57) were collected in 2002-03 at five localities in Utah and Nevada (Utah: Oquirrh Mountains, n = 8; Lookout Pass, n = 4; La Sal National Forest, n= 6, Stansbury Mountains, n = 2; Nevada: vicinity of Austin, n = 37). Bills were measured with calipers and the number of lice on each bird was determined using the body washing method [41]. Panel (**a**) shows the distribution of overhang length (mean of three measurements per bird) across all 57 birds, as well as across the 20 birds (35%) that were infested with lice. Factors other than preening, such as ambient humidity, are known to influence the prevalence of feather lice on Western Scrub-jays [38]. Nevertheless, of the twenty birds that had lice, those with intermediate overhangs had the fewest lice (Fig. **5b**; quadratic regression $R^2 = 0.30$, $P < 0.05$). This intriguing relationship suggests that lice exert stabilizing selection for intermediate overhang length, presumably because intermediate overhangs are best at controlling lice (cf. Clayton *et al.* [125]). An experimental test of this hypothesis is needed.

ten have large numbers of ectoparasites (and their eggs) concentrated around the head and neck [112]. The obvious explanation is that, although birds can preen themselves while standing on one leg, they cannot scratch themselves. Although the precise impact of scratching on ectoparasites has not been measured, scratching is known to kill or damage fleas on domestic chickens (Suter cited in Marshall [5]).

Birds may use scratching to compensate for a lack of other methods of ectoparasite control. The unpaired penguins in Brooke's [131] study spent significantly more time scratching than did paired individuals with access to allopreening. Scratching also appears to compensate for inefficient preening in species with unwieldy bills. Clayton and Cotgreave [105] reported that long-billed species average 16.2% of their grooming time scratching, compared to 2.3% in short-billed species. In a series of paired taxonomic comparisons, long-billed species scratched significantly more than short-billed taxa. In another comparative study, Clayton and Walther [124] investigated the relationship of relative foot length and toenail flange width to the louse loads of Peruvian birds, but neither feature was correlated with louse species richness or abundance.

The efficiency of scratching for ectoparasite control may be enhanced by the presence of a comb-like pectinate claw on the middle toes of some birds (Fig. **6**) [136-138]. But the possible ectoparasite control function of this "louse comb" has long been controversial [139]. Other possible functions include a role in feeding [140], removal of stale powder down from the plumage [141], or straightening of rictal bristles [137, 142]. To our knowledge, however, none of these functional hypotheses, including ectoparasite control, has ever been tested. Even the distribution of the pectinate claw among bird taxa has not been carefully documented.

One of us (BRM) recently examined 1421 study skins for pectinate claws in the collection of the Division of Birds, National Museum of Natural History, Washington DC. At

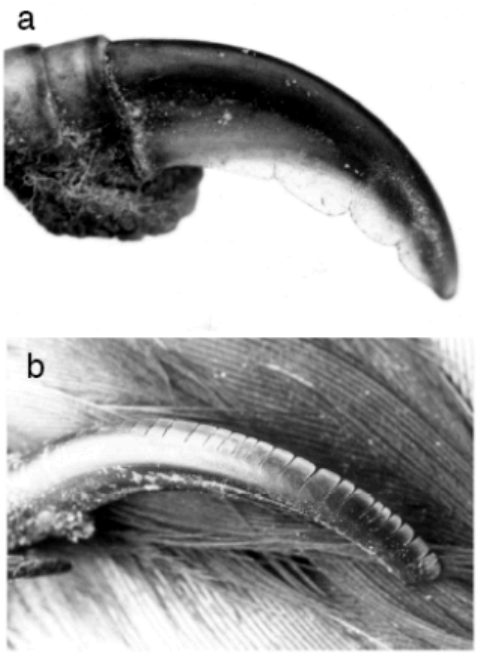

Fig. (6). Variation in the structure of the pectinate claw, ranging from (**a**) the coarsely serrated claw of the American Dipper (*Cinclus mexicanus*) to (**b**) the finely serrated claw of the Magnificent Frigatebird (*Fregata magnificens*). Photos by C. Beittel.

least one representative species from each of 118 (82%) of the 144 bird families recognized by Sibley and Ahlquist [143] was selected (haphazardly), and all of the claws of one male and one female specimen were examined under 6x magnification. Skins of species noted in the literature to have a pectinate claw were also examined [138, 144-146] . A pectinate claw was considered to be present if any portion of any claw was serrate. If a pectinate claw was detected in a given family during the initial survey, at least one representative species from every available genus in this family was subsequently examined to assess within family variation (5 males and 5 females were examined when possible).

Most birds lack a pectinate claw. Only 17 of 118 families contained individuals with pectinate claws (Table **I**), and of the Passeriformes, only dippers (Cinclidae) had them (Fig **6a**). The claw has probably evolved repeatedly, given its scattered distribution across bird families. It is also variable within families; only a minority of genera possess it within most of the 17 families. For example, a pectinate claw is present in one of four genera of Heliornithidae, two of 12 genera of Scolopacidae, four of six genera of Glareolidae, four of 16 genera of Laridae, and one of ten genera of Threskiornithidae (Table **I**).

In addition to within-family variation, we discovered within-species variation in pectinate claws. In 15 species some individuals had the claw, while others lacked it (Table **I**). This intraspecific variation did not appear to be related to the sex, geographic distribution, or season in which the bird was collected. We did not examine variation in relation to the bird's age, but this would also be interesting to explore. The structure of the pectinate claw varied considerably among taxa. Pectinations ranged from scalloping, as in the American Dipper (*Cinclus mexicanus*) (Fig. **6a**), to fine serrations, as in the Magnificent Frigatebird (*Fregata magnificens*) (Fig. **6b**).

Serrations on some pectinate claws are somewhat similar to the teeth of combs designed to remove human head lice. Clay [138] believed that species with pectinate claws might be more efficient at removing lice from the head by scratching. She predicted that birds with pectinate claws would be parasitized by fewer species of head lice than those species without pectinate claws. We tested Clay's hypothesis using an analysis that compared the species richness of head lice on birds with pectinate claws to that of sister taxa without pectinate claws. We selected 14 phylogenetically independent comparisons of bird species with and without pectinate claws. We then asked a louse taxonomist colleague to tally the number of species of head lice known from each species of bird using Price *et al.* [63]. Because louse species richness is influenced by sampling effort, we corrected for this factor as described in Walther *et al.* [147].

Our analysis revealed no significant difference in the number of species of head lice on birds with and without claws (Wilcoxon signed-rank test on residuals, $T = 24.5$, $P = 0.15$). In eight of the 14 comparisons, the species with the pectinate claw had fewer (residual) species of head lice, and in five comparisons the reverse was true (one tie). In retrospect, it is unclear why one should necessarily expect a negative correlation between the pectinate claw and louse species richness, or a positive correlation. If richness decreases on birds that evolve pectinate claws, then selection maintaining the claw would be relaxed, leading to disappearance of the claw. Hence, this comparative analysis is perhaps not the most convincing test of the hypothesis that pectinate claws help to control ectoparasites.

We have also investigated the relationship between louse abundance and pectinate claw morphology within species. We used 24 road-killed Barn Owls (*Tyto alba*) salvaged by colleagues along highways in southern Idaho. We counted the number of teeth on the pectinate claw of each foot (Fig **7a**), and we measured the length and width of each claw's flange. The number of lice on each owl was quantified using "body washing" [41]. Fourteen (58%) of the owls had lice, but one was missing the pectinate claw on one foot. Since we could not be sure whether this was natural, or a consequence of post-mortem road damage, this individual bird was excluded from the analysis.

There was no significant difference in the number of teeth or the length or width of the flange, between infested and uninfested owls ($n = 23$, $df = 1$, $P > 0.27$). Similarly, there was no significant relationship between the abundance of lice on infested owls, and the mean number of teeth per claw (Fig. **7c**). Finally, there was no relationship between louse abundance and mean claw length ($n = 13$, $R^2 = 0.006$, $P = 0.81$), or width ($n = 13$, $R^2 = 0.02$, $P = 0.63$). The results of this study indicate that natural variation in the size and shape of the pectinate claw does not correlate with louse prevalence or intensity, at least in the case of Barn Owls from southern Idaho.

In summary, these comparative and correlational studies indicate that the pectinate claw plays no role in parasite control. However, a more definitive test would be to conduct an experiment in which parasite populations are monitored on birds with normal claws, versus birds from which the pectinations have been removed, perhaps by filing them off. There are several common species that could be used for this experiment, such as Cattle Egrets *(Bubulcus ibis)* (Table **I**).

Bathing

Another form of maintenance behavior practiced by most birds is bathing in water.

Rothschild and Clay [4] wrote, "Bathing in water and dust and the subsequent preening helps the bird to rid itself of parasites." However, we are not aware of any evidence suggesting that water bathing has a detrimental effect on ectoparasites. If anything, it might be expected to have a positive effect, given that high humidity favors ectoparasites ranging from feather lice [37] to bacteria [70]. It is conceivable that substances detrimental to ectoparasites might be dissolved in some water sources, but we know of no support for this speculation.

Dusting

Members of at least a dozen orders of birds are known to engage in dusting (Table **II**), during which fine dirt or sand is ruffled through the plumage [148-150] (Fig. **8**). Dusting appears to remove excess feather oil that can cause matting of plumage [151-153]. It is also thought to help control ectoparasites. Several mechanisms for such control have been proposed, including (1) reducing feather lipids upon which some ectoparasites feed [152]; (2) directly dislodging para

Table I. Occurrence of Pectinate Claws Among 1421 Study Skins of Birds Representing 278 Species in 250 Genera (118 Families, 23 Orders). Species with Pectinate Claws are in Boldface. Presence (+) or Absence (-) of a Maxillary Overhang on the Bill is also Indicated in the Final Column; Lack of a Symbol Means the Species was not Checked for an Overhang. Classification and Nomenclature Follow Sibley and Monroe [252]

Higher Taxa	Bird Species	# With Claw/ # Examined (%)	Bill Overhang Present (+), Absent (-), or Unexamined ( )
Struthioniformes			
Struthionidae	Ostrich, *Struthio camelus*	0/2 (0)	+
Rheidae	Greater Rhea, *Rhea americana*	0/2 (0)	+
Casuariidae	Dwarf Cassowary, *Casuarius bennetti*	0/2 (0)	+
	Emu, *Dromaius novaehollandiae*	0/2 (0)	+
Apterygidae	Brown Kiwi, *Apteryx australis*	0/2 (0)	+
Tinamiformes			
Tinamiidae	Variegated Tinamou, *Crypturellus variegatus*	0/2 (0)	+
	Elegant Crested-Tinamou, *Eudromia elegans*	0/2 (0)	
Craciformes			
Cracidae	Grey-headed Chachalaca, *Ortalis cinereiceps*	0/2 (0)	+
	Blue-knobbed Curassow, *Crax alberti*	0/2 (0)	
Megapodiidae	Brown-collared Brush-turkey, *Talegalla jobiensis*	0/2 (0)	+
Galliformes			
Phasianidae	Green Peafowl, *Pavo muticus*	0/2 (0)	
	Spruce Grouse, *Dendragapus canadensis*	0/2 (0)	+
	Ruffed Grouse, *Bonasa umbellus*	0/2 (0)	
	Wild Turkey, *Meleagris gallopavo*	0/2 (0)	+
Numididae	Crested Guineafowl, *Guttera pucherani*	0/2 (0)	+
Odontophoridae	Northern Bobwhite, *Colinus virginianus*	0/2 (0)	+
Anseriformes			
Anhimidae	Southern Screamer, *Chauna torquata*	0/2 (0)	+
Anatidae	Emperor Goose, *Anser canagica*	0/2 (0)	+
	Common Teal, *Anas crecca*	0/2 (0)	
Turniciformes			
Turnicidae	Barred Buttonquail, *Turnix suscitator*	0/2 (0)	+
Piciformes			
Indicatoridae	Lesser Honeyguide, *Indicator minor*	0/2 (0)	+
Picidae	Black-cheeked Woodpecker, *Melanerpes pucherani*	0/2 (0)	
	Greater Flameback, *Chrysocolaptes lucidus*	0/2 (0)	-
Lybiidae	Green Barbet, *Stactolaema olivacea*	0/2 (0)	+
Ramphastidae	Yellow-eared Toucanet, *Selenidera spectabilis*	0/2 (0)	+
Galbuliformes			

Table 1. Contd….

Higher Taxa	Bird Species	# With Claw/ # Examined (%)	Bill Overhang Present (+), Absent (-), or Unexamined ( )
Galbulidae	Great Jacamar, *Jacamerops aurea*	0/2 (0)	+
Bucconidae	White-whiskered Puffbird, *Malacoptila panamensis*	0/2 (0)	+
Bucerotiformes			
Bucerotidae	Helmeted Hornbill, *Buceros vigil*	0/2 (0)	
	White-crowned Hornbill, *Aceros comatus*	0/2 (0)	+
Upupiformes			
Upupidae	Eurasian Hoopoe, *Upupa epops*	0/2 (0)	+
Phoeniculidae	White-headed Woodhoopoe, *Phoeniculus bollei*	0/2 (0)	+
Trogoniformes			
Trogonidae	Diard's Trogon, *Harpactes diardii*	0/2 (0)	+
Coraciiformes			
Coraciidae	Purple-winged Roller, *Coracias temminckii*	0/2 (0)	+
Leptosomidae	Courol, *Leptosomus discolor*	0/2 (0)	+
Momotidae	Turquoise-browed Motmot, *Eumomota superciliosa*	0/2 (0)	+
Todidae	Broad-billed Tody, *Todus subulatus*	0/2 (0)	+
Dacelonidae	Laughing Kookaburra, *Dacelo novaeguineae*	0/2 (0)	+
Cerylidae	Belted Kingfisher, *Megaceryle alcyon*	0/2 (0)	+
Meropidae	Madagascar Bee-eater, *Merops superciliosus*	0/2 (0)	+
Coliiformes			
Coliidae	Blue-naped Mousebird, *Urocolius macrourus*	0/2 (0)	+
Cuculiformes			
Cuculidae	Large Hawk-cuckoo, *Cuculus sparverioides*	0/2 (0)	+
Opisthocomidae	Hoatzin, *Opisthocomus hoazin*	0/2 (0)	+
Psittaciformes			
Psittacidae	Common Kaka, *Nestor meridionalis*	0/2 (0)	+
Apodiformes			
Apodidae	White-throated Swift, *Aeronautes saxatalis*	0/2 (0)	+
Hemiprocnidae	Grey-rumped Treeswift, *Hemiprocne longipennis*	0/2 (0)	+
Trochiliformes			
Trochilidae	Black-hooded Sunbeam, *Aglaeactis pamela*	0/2 (0)	-
Musophagiformes			
Musophagidae	Knysna Turaco, *Tauraco corythaix*	0/2 (0)	+
Strigiformes			
Tytonidae	**Barn Owl, *Tyto alba***	**12/12 (100)**	+
	Oriental Bay-Owl, *Phodilus badius*	**3/3 (100)**	

Table 1. Contd....

Higher Taxa	Bird Species	# With Claw/ # Examined (%)	Bill Overhang Present (+), Absent (-), or Unexamined ( )
Strigidae	Eastern Screech-Owl, *Otus asio*	0/2 (0)	+
	Great Horned Owl, *Bubo virginianus*	0/2 (0)	
	Short-eared Owl, *Asio flammeus*	0/10 (0)	
Aegothelidae	Australian Owlet-Nightjar, *Aegotheles cristatus*	0/2 (0)	+
Podargidae	Tawny Frogmouth, *Podargus strigoides*	0/10 (0)	+
Batrachostomidae	Philippine Frogmouth, *Batrachostomus septimus*	0/2 (0)	+
Steatornithidae	Oilbird, *Steatornis caripensis*	0/2 (0)	+
Nyctibiidae	Great Potoo, *Nyctibius grandis*	0/2 (0)	+
Eurostopodidae	**Great Eared-Nightjar, *Eurostopodus macrotis***	**10/10 (100)**	+
Caprimulgidae	**Short-tailed Nighthawk, *Lurocalis semitorquatus***	**9/9 (100)**	
	Common Nighthawk, *Chordeiles minor*	**10/10 (100)**	+
	Band-tailed Nighthawk, *Nyctiprogne leucopyga*	**5/5 (100)**	
	Nacunda Nighthawk, *Podager nacunda*	**10/10 (100)**	
	Paraque, *Nyctidromus albicollis*	**10/10 (100)**	
	Common Poorwill, *Phalaenoptilus nuttallii*	**10/10 (100)**	
	Ocellated Poorwill, *Nyctiphrynus ocellatus*	**2/2 (100)**	
	Whip-Poor-Will, *Caprimulgus vociferus*	**10/10 (100)**	
	Standard-winged Nightjar, *Macrodipteryx longipennis*	**2/2 (100)**	
Columbiformes			
Columbidae	Rock Pigeon, *Columba livia*	0/2 (0)	
	Pied Imperial-Pigeon, *Ducula bicolor*	0/2 (0)	+
Gruiformes			
Eurypygidae	Sunbittern, *Eurypyga helias*	0/2 (0)	+
Otididae	Black-bellied Bustard, *Eupodotis melanogaster*	0/2 (0)	+
Gruidae	Common Crane, *Grus grus*	0/2 (0)	+
Heliornithidae	Limpkin, *Aramus guarauna*	0/2 (0)	+
	African Finfoot, *Podica senegalensis*	**4/4 (100)**	
	Masked Finfoot, *Heliopais personata*	0/4 (0)	+
	Sungrebe, *Heliornis fulica*	0/10 (0)	
Psophiidae	Grey-winged Trumpeter, *Psophia crepitans*	0/2 (0)	+
Cariamidae	Red-legged Seriema, *Cariama cristata*	0/2 (0)	+
Rhynochetidae	Kagu, *Rhynochetus jubata*	0/2 (0)	+
Rallidae	King Rail, *Rallus elegans*	0/2 (0)	+
	Giant Coot, *Fulica gigantea*	0/2 (0)	

Table 1. Contd….

Higher Taxa	Bird Species	# With Claw/ # Examined (%)	Bill Overhang Present (+), Absent (-), or Unexamined ( )
Mesitornithidae	Brown Roatelo, *Mesitornis unicolor*	0/2 (0)	+
Ciconiiformes			
Pteroclidae	Lichtenstein's Sandgrouse, *Pterocles lichtensteinii*	0/2 (0)	+
Thinocoridae	Grey-breasted Seedsnipe, *Thinocorus orbignyianus*	0/2 (0)	+
Pedionomidae	Plains-Wanderer, *Pedionomus torquatus*	0/2 (0)	+
Scolopacidae	Common Snipe, *Gallinago gallinago*	0/10 (0)	
	Jack Snipe, *Lymnocryptes minimus*	0/10 (0)	
	Black-tailed Godwit, *Limosa limosa*	**10/12 (83)**	
	Long-billed Curlew, *Numenius americanus*	0/21 (0)	
	Upland Sandpiper, *Bartramia longicauda*	0/10 (0)	
	Greater Yellowlegs, *Tringa melanoleuca*	0/2 (0)	+
	Lesser Yellowlegs, *Tringa flavipes*	0/10 (0)	
	Terek Sandpiper, *Tringa cinerea*	0/10 (0)	
	Black Turnstone, *Arenaria melanocephala*	0/10 (0)	
	Short-billed Dowitcher, *Limnodromus griseus*	0/11 (0)	
	Surfbird, *Aphriza virgata*	**11/17 (65)**	
	Red Knot, *Calidris canutus*	0/10 (0)	
	Ruff, *Philomachus pugnax*	0/10 (0)	
	Red-necked Phalarope, *Phalaropus lobatus*	0/10 (0)	
Rostratulidae	Greater Painted-Snipe, *Rostratula benghalensis*	0/2 (0)	+
Jacanidae	Wattled Jacana, *Jacana jacana*	0/2 (0)	+
Chionididae	Snowy Sheathbill, *Chionis alba*	0/2 (0)	+
Burhinidae	Double-striped Thick-knee, *Burhinus bistriatus*	0/2 (0)	+
	Beach Thick-knee, *Burhinus giganteus*	0/15 (0)	
Charadriidae	Black Oystercatcher, *Haematopus bachmani*	0/2 (0)	-
	American Avocet, *Recurvirostra americana*	0/2 (0)	+
	Killdeer, *Charadrius vociferus*	0/2 (0)	
	Northern Lapwing, *Vanellus vanellus*	0/14 (0)	+
Glareolidae	**Crab Plover, *Dromas ardeola***	**7/10 (70)**	+
	Crocodile-bird, *Pluvianus aegyptius*	0/4 (0)	
	Three-banded Courser, *Rhinoptilus cinctus*	**7/7 (100)**	
	Cream-colored Courser, *Cursorius cursor*	**10/11 (91)**	
	Indian Courser, *Cursorius coromandelicus*	**2/2 (100)**	
	Collared Pratincole, *Glareola pratincola*	**10/19 (53)**	+
	Oriental Pratincole, *Glareola maldivarum*	**10/12 (83)**	

Table 1. Contd….

Higher Taxa	Bird Species	# With Claw/ # Examined (%)	Bill Overhang Present (+), Absent (-), or Unexamined ( )
	Australian Pratincole, *Stiltia isabella*	0/5 (0)	
Laridae	South Polar Skua, *Catharacta maccormicki*	0/12 (0)	
	Pomarine Jaeger, *Stercorarius pomarinus*	0/2 (0)	+
	Long-tailed Jaeger, *Stercorarius longicaudus*	0/10 (0)	
	Black Skimmer, *Rynchops niger*	0/12 (0)	-
	Laughing Gull, *Larus atricilla*	0/12 (0)	+
	Sabine's Gull, *Xema sabini*	0/10 (0)	
	Gull-billed Tern, *Sterna nilotica*	0/10 (0)	
	Caspian Tern, *Sterna caspia*	0/10 (0)	
	Black-naped Tern, *Sterna sumatrana*	**9/10 (0)**	
	Black Tern, *Chlidonias niger*	0/10 (0)	
	Large-billed Tern, *Phaetusa simplex*	0/10 (0)	
	Brown Noddy, *Anous stolidus*	**16/20 (80)**	
	Black Noddy, *Anous minutus*	0/2 (0)	
	Blue Noddy, *Procelsterna cerulea*	**9/10 (90)**	
	Common White-Tern, *Gygis alba*	0/10 (0)	
	Inca Tern, *Larosterna inca*	**6/7 (86)**	
	Dovekie, *Alle alle*	0/10 (0)	
	Razorbill, *Alca torda*	0/2 (0)	+
	Pigeon Guillemot, *Cepphus columba*	0/10 (0)	
	Tufted Puffin, *Fratercula cirrhata*	0/2 (0)	
Accipitridae	Osprey, *Pandion haliaetus*	0/2 (0)	+
	Mississippi Kite, *Ictinia mississippiensis*	0/2 (0)	+
	Cooper's Hawk, *Accipiter cooperii*	0/2 (0)	
Sagittariidae	Secretary-Bird, *Sagittarius serpentarius*	0/2 (0)	+
Falconidae	Crested Caracara, *Polyborus plancus*	0/2 (0)	+
	American Kestrel, *Falco sparverius*	0/11 (0)	
Podicipedidae	**White-tufted Grebe, *Rollandia rolland***	**7/8 (88)**	
	Australasian Grebe, *Tachybaptus novaehollandiae*	**7/7 (100)**	
	Least Grebe, *Tachybaptus dominicus*	**10/10 (100)**	
	Pied-billed Grebe, *Podilymbus podiceps*	**12/12 (100)**	+
	Western Grebe, *Aechmophorus occidentalis*	**12/12 (100)**	
Phaethontidae	Red-tailed Tropicbird, *Phaethon rubricauda*	0/12 (0)	+
	White-tailed Tropicbird, *Phaethon lepturus*	0/10 (0)	
Sulidae	**Northern Gannet, *Morus bassanus***	**9/9 (100)**	+

Table 1. Contd....

Higher Taxa	Bird Species	# With Claw/ # Examined (%)	Bill Overhang Present (+), Absent (-), or Unexamined ( )
	Masked Booby, *Sula dactylatra*	**2/2 (100)**	
	Brown Booby, *Sula leucogaster*	**11/11 (100)**	
Anhingidae	**Anhinga, *Anhinga anhinga***	**10/10 (100)**	
	Oriental Darter, *Anhinga melanogaster*	**2/2 (100)**	-
Phalacrocoracidae	**Little Cormorant, *Phalacrocorax niger***	**2/2 (100)**	
	Brandt's Cormorant, *Phalacrocorax penicillatus*	**10/10 (100)**	
	Neotropic Cormorant, *Phalacrocorax brasilianus*	**2/2 (100)**	+
	Double-crested Cormorant, *Phalacrocorax auritus*	**10/10 (100)**	
Ardeidae	**Great Blue Heron, *Ardea herodias***	**12/12 (100)**	-
	Cattle Egret, *Bubulcus ibis*	**13/13 (100)**	
	Chinese Pond-Heron, *Ardeola bacchus*	**10/10 (100)**	
	Yellow-crowned Night-Heron, *Nyctanassa violacea*	**10/10 (100)**	
	Black-crowned Night-Heron, *Nycticorax nycticorax*	**10/10 (100)**	
	Boat-billed Heron, *Cochlearius cochlearius*	**10/10 (100)**	
	Bare-throated Tiger-Heron, *Tigrisoma mexicanum*	**10/10 (100)**	
	White-crested Bittern, *Tigriornis leucolophus*	**2/2 (100)**	
	Zigzag Heron, *Zebrilus undulatus*	**1/1 (100)**	
	Stripe-backed Bittern, *Ixobrychus involucris*	**4/4 (100)**	
	Great Bittern, *Botaurus stellaris*	**8/8 (100)**	
Scopidae	**Hamerkop, *Scopus umbretta***	**10/10 (100)**	+
Phoenicopteridae	Greater Flamingo, *Phoenicopterus ruber*	0/12 (0)	+
Threskiornithidae	White Ibis, *Eudocimus albus*	0/10 (0)	
	White-faced Ibis, *Plegadis chihi*	**12/12 (100)**	
	Plumbeous Ibis, *Theristicus caerulescens*	0/5 (0)	
	Buff-necked Ibis, *Theristicus caudatus*	0/9 (0)	
	Green Ibis, *Mesembrinibis cayennensis*	0/10 (0)	
	Hadada Ibis, *Bostrychia hagedash*	0/12 (0)	
	Wattled Ibis, *Bostrychia carunculata*	0/5 (0)	
	Spot-breasted Ibis, *Bostrychia rara*	0/1 (0)	
	Bald Ibis, *Geronticus calvus*	0/2 (0)	
	Sacred Ibis, *Threskiornis aethiopicus*	0/10 (0)	
	Straw-necked Ibis, *Threskiornis spinicollis*	0/5 (0)	
	White-shouldered Ibis, *Pseudibis davisoni*	0/13 (0)	+
	Giant Ibis, *Pseudibis gigantea*	0/4 (0)	
	Crested Ibis, *Nipponia nippon*	0/6 (0)	

Table 1. Contd....

Higher Taxa	Bird Species	# With Claw/ # Examined (%)	Bill Overhang Present (+), Absent (-), or Unexamined ( )
	Roseate Spoonbill, *Ajaia ajaja*	0/12 (0)	
Pelecanidae	**Shoebill, *Balaeniceps rex***	**2/2 (100)**	+
	American White Pelican, *Pelecanus erythrorhynchos*	**3/11 (27)**	
	Brown Pelican, *Pelecanus occidentalis*	**11/11 (100)**	+
Ciconiidae	Turkey Vulture, *Cathartes aura*	0/2 (0)	+
	Andean Condor, *Vultur gryphus*	0/2 (0)	
	White Stork, *Ciconia ciconia*	0/9 (0)	+
	Lesser Adjutant, *Leptoptilos javanicus*	0/2 (0)	
Fregatidae	**Magnificent Frigatebird, *Fregata magnificens***	**12/12 (100)**	+
	Great Frigatebird, *Fregata minor*	**10/10 (100)**	
Spheniscidae	Gentoo Penguin, *Pygoscelis papua*	0/12 (0)	+
	Jackass Penguin, *Spheniscus demersus*	0/2 (0)	
Gaviidae	Red-throated Loon, *Gavia stellata*	0/7 (0)	
	Arctic Loon, *Gavia arctica*	0/6 (0)	+
	Common Loon, *Gavia immer*	0/12 (0)	
	Yellow-billed Loon, *Gavia adamsii*	0/4 (0)	
Procellariidae	Southern Fulmar, *Fulmarus glacialoides*	0/2 (0)	+
	Juan Fernandez Petrel, *Pterodroma externa*	0/2 (0)	
	Common Diving-Petrel, *Pelecanoides urinatrix*	0/2 (0)	+
	Black-footed Albatross, *Diomedea nigripes*	0/2 (0)	+
	Light-mantled Albatross, *Phoebetria palpebrata*	0/2 (0)	
	Leach's Storm-Petrel, *Oceanodroma leucorhoa*	0/2 (0)	+
Passeriformes			
Acanthisittidae	Rifleman, *Acanthisitta chloris*	0/2 (0)	+
Pittidae	Ivory-breasted Pitta, *Pitta maxima*	0/2 (0)	+
Eurylaimidae	Dusky Broadbill, *Corydon sumatranus*	0/2 (0)	+
Philepittidae	Velvet Asity, *Philepitta castanea*	0/2 (0)	+
Tyrannidae	Highland Elaenia, *Elaenia obscura*	0/2 (0)	+
	Black-necked Red-Cotinga, *Phoenicircus nigricollis*	0/2 (0)	+
	Rufous-tailed Plant-cutter, *Phytotoma rara*	0/2 (0)	+
	Sharpbill, *Oxyruncus cristatus*	0/2 (0)	+
	Long-tailed Manakin, *Chiroxiphia linearis*	0/2 (0)	+
Thamnophilidae	Black-backed Antshrike, *Sakesphorus melanonotus*	0/2 (0)	+
Furnariidae	Azara's Spinetail, *Synallaxis azarae*	0/2 (0)	+
	Long-billed Woodcreeper, *Nasica longirostris*	0/2 (0)	+

Table 1. Contd....

Higher Taxa	Bird Species	# With Claw/ # Examined (%)	Bill Overhang Present (+), Absent (-), or Unexamined ( )
Rhinocryptidae	Moustached Turca, *Pteroptochos megapodius*	0/2 (0)	+
Climacteridae	White-throated Treecreeper, *Cormobates leucophaeus*	0/2 (0)	+
Menuridae	Superb Lyrebird, *Menura novaehollandiae*	0/2 (0)	+
	Rufous Scrub-bird, *Atrichornis rufescens*	0/2 (0)	+
Ptilonorhynchidae	Green Catbird, *Ailuroedus crassirostris*	0/2 (0)	+
Meliphagidae	Wattled Honeyeater, *Foulehaio carunculata*	0/2 (0)	+
Irenidae	Golden-fronted Leafbird, *Chloropsis aurifrons*	0/2 (0)	+
Vireonidae	Yellow-throated Vireo, *Vireo flavifrons*	0/2 (0)	+
Corvidae	Daurian Jackdaw, *Corvus dauuricus*	0/2 (0)	+
	Raggiana Bird-of-Paradise, *Paradisaea raggiana*	0/2 (0)	+
	Grey Currawong, *Strepera versicolor*	0/2 (0)	+
	Dusky Wood-Swallow, *Artamus cyanopterus*	0/2 (0)	+
	Black-hooded Oriole, *Oriolus xanthornus*	0/2 (0)	+
	Bar-bellied Cuckoo-Shrike, *Coracina striata*	0/2 (0)	+
	Square-tailed Drongo, *Dicrurus ludwigii*	0/2 (0)	+
	Magpie-Lark, *Grallina cyanoleuca*	0/2 (0)	+
	White Helmetshrike, *Prionops plumatus*	0/2 (0)	+
	Rufous Vanga, *Schetba rufa*	0/2 (0)	+
	Coral-billed Nuthatch, *Hypositta corallirostris*	0/2 (0)	+
Callaeatidae	Kokako, *Callaeas cinerea*	0/2 (0)	+
Bombycillidae	Palmchat, *Dulus dominicus*	0/2 (0)	+
	Grey Silky-Flycatcher, *Ptilogonys cinereus*	0/2 (0)	+
	Cedar Waxwing, *Bombycilla cedrorum*	0/2 (0)	+
Cinclidae	**White-throated Dipper, *Cinclus cinclus***	**3/15 (20)**	
	Brown Dipper, *Cinclus pallasii*	**3/22 (14)**	
	American Dipper, *Cinclus mexicanus*	**10/20 (50)**	+
Muscicapidae	Swainson's Thrush, *Catharus ustulatus*	0/20 (0)	
	White-bellied Short-wing, *Brachypteryx major*	0/2 (0)	+
Sturnidae	Red-winged Starling, *Onychognathus morio*	0/2 (0)	+
	Grey Catbird, *Dumetella carolinensis*	0/2 (0)	+
Sittidae	Wood Nuthatch, *Sitta europaea*	0/2 (0)	+
	Wallcreeper, *Tichodroma muraria*	0/2 (0)	+
Certhiidae	Eurasian Tree-Creeper, *Certhia familiaris*	0/2 (0)	+
	Cactus Wren, *Campylorhynchus brunneicapillus*	0/2 (0)	+
Paridae	African Penduline-Tit, *Anthoscopus caroli*	0/2 (0)	+

Table 1. Contd....

Higher Taxa	Bird Species	# With Claw/ # Examined (%)	Bill Overhang Present (+), Absent (-), or Unexamined ( )
	Carolina Chickadee, *Parus carolinensis*	0/2 (0)	+
Aegithalidae	Bushtit, *Psaltriparus minimus*	0/2 (0)	+
Hirundinidae	Caribbean Martin, *Progne dominicensis*	0/2 (0)	+
Pycnonotidae	Red-whiskered Bulbul, *Pycnonotus jocosus*	0/2 (0)	+
Zosteropidae	White-breasted White-eye, *Zosterops abyssinicus*	0/2 (0)	+
Sylviidae	Gray's Warbler, *Locustella fasciolata*	0/2 (0)	+
	Ferruginous Babbler, *Trichastoma bicolor*	0/2 (0)	+
	Brown Parrotbill, *Paradoxornis unicolor*	0/2 (0)	+
	Stripe-sided Rhabdornis, *Rhabdornis mysticalis*	0/2 (0)	+
	Wrentit, *Chamaea fasciata*	0/2 (0)	+
Alaudidae	Austral-Asian Lark, *Mirafra javanica*	0/2 (0)	+
Nectariniidae	Yellow-sided Flowerpecker, *Dicaeum aureolimbatum*	0/2 (0)	+
	Purple-throated Sunbird, *Nectarinia sperata*	0/2 (0)	+
Passeridae	Russet Sparrow, *Passer rutilans*	0/2 (0)	+
	Yellow Wagtail, *Motacilla flava*	0/2 (0)	+
	Alpine Accentor, *Prunella collaris*	0/2 (0)	+
	White-breasted Negrofinch, *Nigrita fusconota*	0/2 (0)	+
Fringillidae	Iiwi, *Vestiaria coccinea*	0/2 (0)	+
	Nashville Warbler, *Vermivora ruficapilla*	0/2 (0)	+
	Grass-green Tanager, *Chlorornis riefferii*	0/2 (0)	+
	Swallow Tanager, *Tersina viridis*	0/2 (0)	+
	Grey-bellied Flower-piercer, *Diglossa carbonaria*	0/2 (0)	+
	Rose-breasted Grosbeak, *Pheucticus ludovicianus*	0/2 (0)	+
	Audubon's Oriole, *Icterus graduacauda*	0/2 (0)	+

sites [154, 155]; (3) plugging parasite spiracles (breathing pores), leading to poor respiration; and (4) abrading the cuticle, leading to desiccation [132,149]. Desiccation is an intriguing possibility, given that inert dusts, such as volcanic ash, are known to kill insects by abrading their cuticles [156, 157]. Surprisingly, however, no rigorous test of this hypothesis has been conducted. Indeed, to our knowledge, no direct test of the impact of dusting behavior on ectoparasites has ever been performed. Such a study is feasible because many birds dust readily in captivity [153, 158-161]. It should be possible to "seed" parasite-free birds with identical numbers of parasites, such as feather lice, and then provide experimental birds with containers of dust. It might even be possible elicit dusting behavior in control birds by providing them a substance that is known to be harmless to ectoparasites.

Sunning

At least 50 families of birds are known to adopt stereotyped postures and expose themselves to solar radiation, which is known as "sunning" [162] (Fig. **9**). Sunning is thought to control ectoparasites, either by killing them directly or by increasing their vulnerability to preening as they try to escape from the heat [163]. Sunning has intriguing parallels to "behavioral fever," which is when ectothermic animals exploit warm microclimates to combat parasites [164-166]. For example, in response to bacterial infections, Desert Iguanas (*Dipsosaurus dorsalis*) move to warm microclimates and generate a 2°C fever, which increases their survival [167]. Goldfish (*Carassius auratus*) increase their survival in the face of bacterial infection by frequenting warm water, which elevates their body temperature [168].

In warm environments many birds sun to the point of apparent hyperthermia [163, 169-172]. Some birds sun when it is hottest outside, not when it is coolest, suggesting that such sunning has little or nothing to do with conserving body heat. For example, Black Noddies (*Anous minutus*) in tropical Australia sun most frequently during periods of high temperature rather than low temperature [163], and several species of swallows sun only on hot summer days [172 -

Fig. (7). Barn Owls (*Tyto alba*) have a pectinate claw on their middle toe (**a**, photo by S. Bush), which is used in scratching (**b**, photo by Mike Read, naturepl.com). One adaptive function of this claw may be to remove ectoparasites. We studied natural variation in louse load and pectinate claw morphology of Barn Owls from Southern Idaho. (**c**) The relationship between the number of lice and the mean number of teeth per claw is not significant ($n = 13$, $R^2 = 0.01$, $P = 0.72$).

175]. Both noddies and swallows pant during these sunning episodes, indicating heat stress.

Table II. Examples of Birds Known to Dust; from Moyer and Clayton [22]

STRUTHIONIFORMES	**STRIGIFORMES**
STRUTHIONIDAE	STRIGIDAE
Ostrich *(Struthio)*	Owl
RHEIFORMES	**CAPRIMULGIFORMES**
RHEIDAE	CAPRIMULGIDAE
Rhea *(Rhea)*	Nightjar
FALCONIFORMES	**COLIIFORMES**
ACCIPITRIDAE	COLIIDAE
Hawk	Mousebird
FALCONIDAE	**CORACIIFORMES**
Falcon	MOMOTIDAE
GALLIFORMES	Motmot
PHASIANIDAE	MEROPIDAE
Grouse *(Lagopus)*	Bee-eater
Bobwhite *(Colinus)*	CORACIIDAE
Fowl *(Gallus)*	Roller
Quail *(Coturnix)*	UPUPIDAE
Partridge *(Alectoris)*	Hoopoe
Pheasant *(Chrysolophus, Phasianus)*	BUCEROTIDAE
GRUIFORMES	Hornbill
TURNICIDAE	**PASSERIFORMES**
Buttonquail	ALAUDIDAE
CARIAMIDAE	Lark
Seriema	TROGLODYTIDAE
OTIDIDAE	Wren
Bustard	TIMALIIDAE
CHARADRIIFORMES	Wrentit *(Chamaea)*
THINOCORIDAE	**EMBERIZIDAE**
Seedsnipe	Sparrow (Spizella, Pooecetes)
COLUMBIFORMES	ICTERIDAE
COLUMBIDAE	Grackle *(Quiscalus)*
Dove	PLOCEIDAE
PTEROCLIDIDAE Sandgrouse	Sparrow (Passer, Petronia, Montifringilla)
	GRALLINIDAE
	Chough *(Corcorax)*

Fig. (8). Southern Ground-hornbill (*Bucorvus leadbeateri*) dusting itself. Photo by T. Laman (naturepl.com).

Fig. (9). White-rumped Shama (*Copsychus malabaricus*) sunning itself. Photo by Michael Luckett (fotolia.com).

Two lines of evidence are consistent with the hypothesis that sunning helps control ectoparasites. Blem and Blem [172] compared the rate of sunning in Violet-green Swallows (*Tachycinete thalassina*); experimentals were fumigated to remove ectoparasites, while controls were not. Fumigated birds sunned less frequently than controls [172], suggesting that the motivation to sun decreases with a reduction in ectoparasite load. Moyer and Wagenbach [163] exposed lice, placed on model Black Noddy wings, to sun and shade. The duration of exposure was typical of those sunning bouts, and the temperature of the model wings did not exceed that of the wings of actual sunning noddies (temperature was measured from a distance with an infrared thermometer). Significantly more lice died in the sun than in the shade.

Although this work suggests that one adaptive function of sunning is ectoparasite control, additional research is needed to determine exactly how effective sunning is for controlling different parasites, and under different conditions. For example, it would be interesting to explore whether sunning by birds with dark plumage is more effec-

tive than sunning by birds with light plumage. Preliminary work by one of us (BRM) indicates that dark feathers heat up more rapidly, and to a higher temperature, than white feathers when exposed to the sun. Furthermore, Rock Pigeon wing lice abandon interbarb refuges of dark feathers sooner than those on white feathers when exposed sunlight. It is tempting to speculate that one cost associated with the evolution of light colored plumage might be that light colored birds have more difficulty controlling ectoparasites by sunning.

Anointing

Another hypothesized defense against ectoparasites is anointing behavior, during which birds and mammals "...apply scent-laden materials to their integument" [25]. A particularly intriguing form of anointing is "anting" behavior, during which birds crush and smear ants on their feathers (active anting), or allow ants to crawl through the plumage (passive anting) [176-181] (Fig. **10**). Anting has been reported in over 200 bird species, most of them Passeriformes [24, 182, 181]. The fact that birds ant exclusively with ants that secrete formic acid, or other pungent fluids, suggests that anting may kill or deter ectoparasites.

Among the most compelling observations suggesting a role of anting in parasite control is Dubinin's [183] account of anting Meadow Pipits (*Anthus pratensis*) (cited in Kelso and Nice, [179]). Dubinin observed four pipits grasping Wood Ants (*Formica rufa*) in their bills and rubbing them through their plumage. He collected these birds shortly thereafter and examined them along with several other pipits that had not been seen anting. The wing feathers of the anting birds were splotched with liquid that Dubinin presumed to be formic acid. Feather mites (*Pterodectes* spp.) on these birds were actively moving across the feathers, and a large proportion of the mites in the moist regions of the feathers were dead. In contrast, mites on the four non-anting birds were positioned between the feather barbs and were undisturbed. More than 25% (163 of 642) of live mites taken

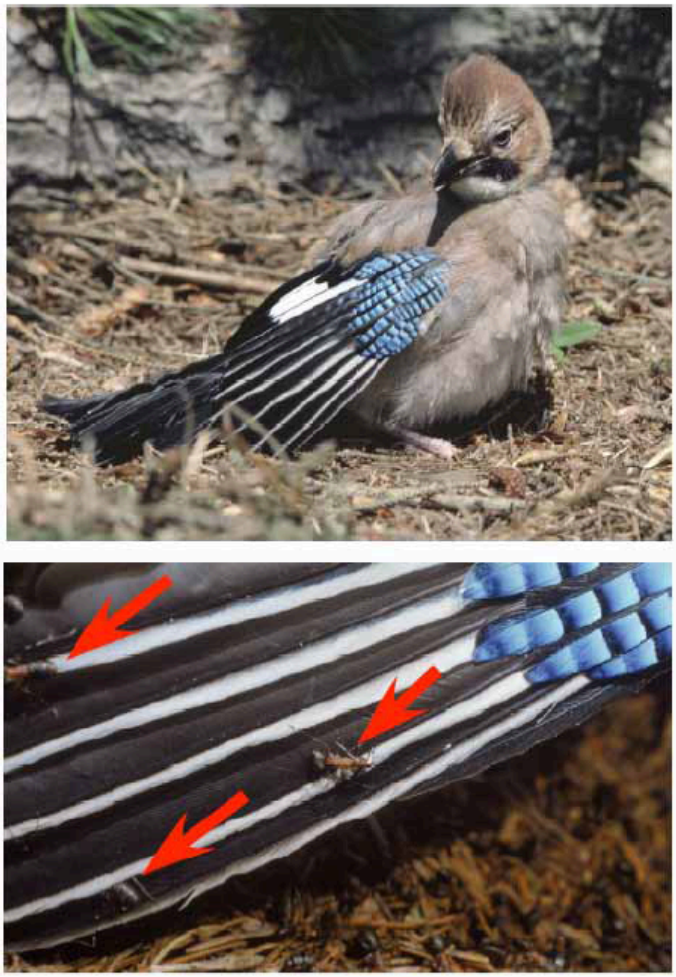

Fig. (10). Jay (*Garrulus glandarius*) anting. Ants (arrows) crawling on the primaries. Photos by A. Cooper (naturepl.com).

from the anting birds died within 12 hr, compared with less than 1% (5 of 758) of those taken from the non-anting birds. Dubinin's observations are consistent with the hypothesis that anting helps control ectoparasites. However, Dubinin's work certainly does not represent a rigorous test of the hypothesis.

Clayton and Wolfe [181] provided a brief synopsis of the results of a field experiment designed to test the impact of anting by European Starlings *(Sturnus vulgaris)* on feather mites and lice. The experiment was conducted using 32 wild-caught birds, half of which were placed in cages (0.8x0.8x0.9 m) directly over natural Wood Ant *(Formica rufa)* trails, while the other half (controls) were placed in identical cages adjacent to ant trails ([184]; Bennett *et al.* unpublished ms). The bottomless cages allowed birds direct access to ants on the ground. The lower portions of the cages were coated with Fluon™, a Teflon-coated liquid that dries to a film ants cannot cross, thus preventing them from swarming up the cage and disturbing the bird. Experimental birds (over ant trails) were observed in frequent anting behavior over the course of the field trials, which lasted three days (birds were removed from the field enclosures at night). By the end of the field trials, experimental birds had plumage that smelled strongly of formic acid. In contrast, control birds did not have access to ant trails and they seldom engaged in anting-like behavior. They did not smell of formic acid at the end of the field trials.

Ectoparasite abundance was quantified on all birds using the visual examination method [41] both the day before field trials started, and again three days after the conclusion of the field trials. The three-day interval allowed birds time to preen dead or damaged ectoparasites, while allowing parasites time to return to normal plumage microhabitats prior to the second visual census. All feather mites visible on each primary and secondary feather of each outstretched wing, as well as mites on the tail feathers, were counted with the aid of a 2x magnifying headset. All of the mites were *Pteronyssoides truncates*. Lice were quantified by tallying only those observed during timed visual counts of specific body regions, including the crown, face, gulum, breast, pectoral region, nape, back (60 s each), as well as the flank and rump (30 s each). Four species of lice were observed: *Menacanthus eurysternus*, *Myrsidea cucullaris*, *Brueelia nebulosa*, and *Sturnidoecus sturni*. Parasite counts were done "blind" to treatment by using Vicks™ Vaporub in the nostrils of the person doing the parasite counts. A few birds escaped or died over the course of the experiment, which left complete data sets for 25 of the 32 birds (14 experimentals and 11 controls).

Despite the fact that experimental birds anted extensively over the three day field trials, there was no significant impact of anting on mites (Fig. **11a**), nor on lice (Fig. **11b**). Since the post-treatment census occurred three days after the final day of formic acid exposure, there should have been ample time for any effect of acid on parasites to occur. *In vitro* studies show that formic acid kills more than 90% of ectoparasites within 15 minutes [177]. In addition to comparing the number of ectoparasites on birds, the condition of all of the lice was noted, as well as a haphazard sample of 25 mites on each wing of each of 14 birds (under magnification). All of the parasites appeared to be in good condition.

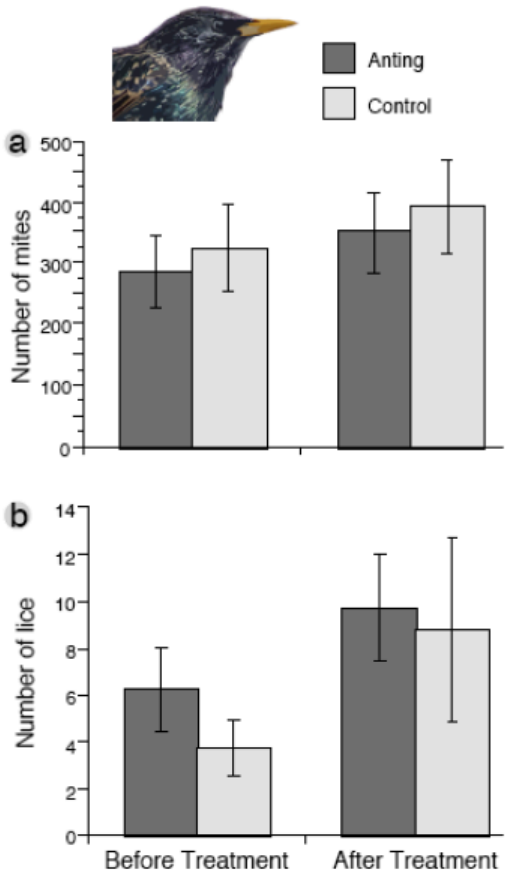

Fig. (11). Mean (± SE) number of (**a**) feather mites and (**b**) lice on European Starlings (*Sturnus vulgaris*) before and after experimental birds were allowed to engage in anting behavior. There was no significant relationship between anting and either mite or louse loads.

These observations indicate that anting has little or no effect on mites or lice, at least on starlings. Interestingly, the number of mites and lice actually increased between the first and second visual censuses, presumably as a result of improvement on the part of the observer, and/or displacement of ectoparasites from refugia where they may have been hiding prior to the initial census and field procedures. It would be worthwhile repeating this experiment using birds that are euthanized and washed after the experiment in order to obtain more accurate estimates of total ectoparasite abundance [41].

Ehrlich *et al.* [185] proposed that anting behavior helps control harmful plumage bacteria or fungi. In a series of inhibition trials, Revis and Waller [186] tested polar and non-polar ant secretions, as well as pure formic acid, for bactericidal and fungicidal effects. Although the formic acid strongly inhibited all bacteria and fungal hyphae tested, concentrations of formic acid approximating those actually found in the bodies of formicine ants did not have an effect.

There was also no detectable effect of hexane ant-chemical extracts, nor ant suspensions in deionized water, on plumage microbes. These results suggest that anting is unlikely to control microbes. Nevertheless, an "*in vivo*" experiment, analogous to the one with starlings described above, is needed for a more definitive test.

Birds also anoint themselves with a bizarre list of other items, including millipedes [187, 188], caterpillars [189], garlic snails [190], bombardier beetles [191], citrus fruits [192, 193, 194], walnut juice [195], flowers [196, 197], lawn chemicals [198] and even mothballs placed in gardens to repel vegetarian pests [199-201]. Many of these items reportedly have anti-parasite properties [201], but few careful tests have been carried out.

Clayton and Vernon [194] performed one such test. The authors observed a Common Grackle (*Quiscalus quiscula*) anointing itself with half a lime fruit. The bird pecked at the fruit repeatedly, then preened itself while holding pieces of lime in its bill. The authors subsequently tested the effect of lime on pigeon lice in the lab. Although lime juice had no effect, exposure to vapor from the lime rind rapidly killed the lice. This result is not surprising, given that lime peel contains D-limonene, a monoterpene present at high concentrations in the peel oil of many citrus fruits, and which is known to be toxic to a wide variety of arthropods [194]. Nevertheless, the hypothesis that birds use citrus peel, or any of the other substances listed above, as a means for actually controlling their ectoparasites is still in need of *in vivo* testing. Experiments using parasitized captive birds engaging in anointing behavior would be informative.

Cosmetic behavior

At least 13 bird families are known to apply "cosmetic" substances to their bodies [202]. The function of this cosmetic behavior is largely unknown, but some examples suggest that the behavior may help combat ectoparasites. For example, Bearded Vultures (*Gypaetus barbatus*) stain their plumage with soils that are rich in iron oxide Fig. (**12**), [203, 204]. They either rub their plumage in dry red soil, or rub damp red soil into their plumage following a bath. Vultures spend as much as an hour applying the soil [204]. Captive Bearded Vultures return to their nests following episodes of soil bathing and rub their newly stained feathers on eggs and offspring [205]. Frey and Roth-Callies [203] [cited in Negro *et al.* [204]] tested for an effect of iron oxide on lice, but there was no significant difference in the survival of lice exposed to a suspension containing iron oxide vs. water controls. Arlettaz *et al.* [205] argued that, since Bearded Vultures are often the last species to feed upon carcasses, they may also be exposed to dangerous quantities of bacteria left behind by earlier scavengers. The oxidative properties in iron oxide rich soils may reduce the negative effects of such bacteria on egg development and nestling growth [205]. However, experimental tests are needed to investigate the effects of iron oxides on bacterial strains.

In a recent review of cosmetic coloration, Delhey and colleagues [202] describe two other kinds of cosmetic behavior that might deter ectoparasites. Shortly before breeding, the Japanese Crested Ibis (*Nipponia nippon*) secretes a black substance from the skin of its head and neck, which is then preened into the plumage [206]; whether this substance has

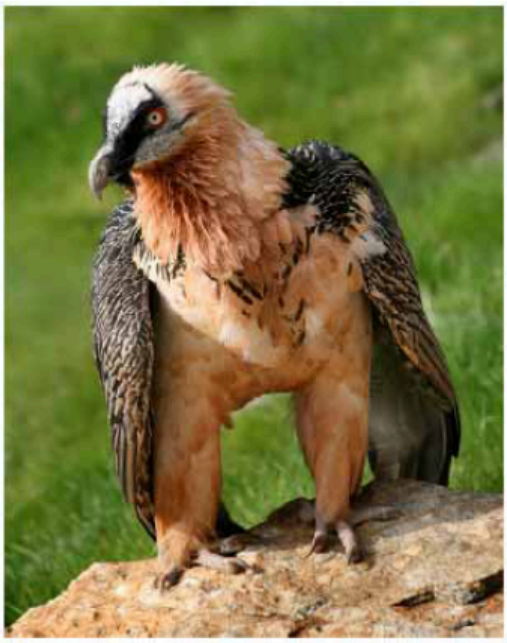

Fig. (12). Bearded vultures (*Gypaetus barbatus*) stain their plumage with soil rich in iron oxide; captive birds without access to such soil have white underparts. Photo by Richard Bartz (commons.wikimedia.org).

any effect on ectoparasites has not been tested. Other species of birds, such as Cinnamon Bitterns (*Ixobrychus cinnamomeus*) and Night Herons (*Nycticorax nycticorax*), apply powder down - specialized feathers that degrade into a powder - to their head and neck regions [207, 208]. The powder down alters the color of these regions, suggesting an intraspecific signaling function. However, since these are also the areas that the bird cannot reach with its bill to preen, it is conceivable that the application of substances to these regions could also help deter parasites. No test of this hypothesis has been conducted.

NEST MAINTENANCE BEHAVIOR

In addition to combating ectoparasites on their bodies, birds must defend themselves from parasites in their nests. Parasites such as fleas, flies, true bugs, and some mites spend portions of their life cycle in the nest material and make brief forays onto nestlings and parents to feed [5]. It is not uncommon for such parasites to kill nestlings or fledglings [45, 209]. Birds have several kinds of nest maintenance behavior that may deter ectoparasites.

Territoriality and Colony Size

Parasite transmission is often more efficient in dense host populations [210]. For this reason, antisocial behavior, such as territoriality, may provide benefits in terms of defense against ectoparasites [211]. Similarly, in colonial species, nesting in smaller colonies can help control ectoparasites because parasite load is proportional to colony size [212].

Nest Site Avoidance

The most effective defense against nest parasites may be to simply avoid them in the first place. A number of studies have shown that birds can detect and avoid nesting (and roosting) sites containing ectoparasites [24, 212-221]. For example, Oppliger *et al.* [214] experimentally investigated the effects of the hematophagous Hen Flea (*Ceratophyllus gallinae*) on nest-site choice in the Great Tit *(Parus major)*. When offered a choice between adjacent nest boxes, one flea-infested and one flea-free, significantly more Great Tits chose parasite-free boxes.

Cliff Swallows (*Hirundo pyrrhonota*) show a similar preference for uninfested nests. Brown and Brown [212] noted that during the early spring, overwintering fleas (*Ceratophyllus celsus*) and Swallow Bugs (*Oeciacus vicarius*) congregate in plain view at the entrances of old swallow nests. This location is a good position from which to infest swallows that enter the nest, or even swallows that come too close to inspect the nest opening, allowing the fleas to leap onto such birds. When they return from the wintering grounds, Cliff Swallows often hover a few centimeters in front of old nests, rather than entering them. This behavior appears to allow the birds to safely inspect the nest opening for ectoparasites [212].

Another way in which birds can avoid ectoparasites is by choosing to breed when fewer ectoparasites are present at nest sites. For example, Great Tits delay reproduction to minimize infestations by Hen Fleas [222], which - like swallow fleas - overwinter in the nest cavity. If a host does not use the cavity, the fleas emigrate [223]. Hence, by delaying reproduction, birds can reduce exposure to fleas. In an experimental test of this delayed-reproduction hypothesis, Oppliger *et al.* [214] found that Great Tits whose nest boxes were infested with fleas started laying eggs 11 days later than birds occupying uninfested nest boxes.

Nest Sanitation

In some cases, birds are known to engage in nest "sanitation" behavior [24]. Female Great Tits and Blue Tits (*Parus caeruleus*) exhibit this behavior, which Christe *et al.* [224] described as "a period of active search with the head dug into the nest material." It is unclear whether this kills or simply disperses ectoparasites, but female Great Tits devote significantly more time to sanitation in flea-infested nests than in uninfested nests [224]. Similarly, female Blue Tits spend more time in sanitation of nests infested with blowfly (*Protocalliphora*) larvae [225] or fleas [226] than in uninfested nests [225]. Another form of nest sanitation is to clean out nests that have been used before; for example, male House Wrens (*Troglodytes aedon*) remove old nest material from their nest boxes prior to each reproductive bout. Pacejka *et al.* [227] showed that this behavior effectively reduces the abundance of mites (*Dermanyssus*) in the nest.

Nest Fumigation

An interesting purported adaptation for controlling ectoparasites in nests is the use of aromatic vegetation to fumigate the nest [24, 25, 73, 181, 228]. Clark and Mason [229] showed that European Starlings (*Sturnus vulgaris*) select species of plants that contain volatile chemicals with antibacterial and insecticidal properties. The authors found that the hatching success of lice (*Menacanthus* sp.) and the growth of several strains of bacteria (*Streptococcus aurealis, Staphylococcus epidermis*, and *Pseudomonas aeruginosa*) were significantly reduced when exposed to volatiles from plants preferred by starlings, compared to a random sample of nearby vegetation. A subsequent study showed that emergence of a mite (*Ornithonyssus sylviarum*) was significantly decreased when wild carrot (*Daucus carota*) or fleabane (*Erigeron philadelphicus*) was added to the nesting material [230].

A recent study of Bonelli's Eagles (*Hieraaetus fasciatus*) showed that nests with higher percentages of pine greenery had fewer blow fly larvae (*Protocalliphora*) and higher host breeding success [231]. The results of this observational study are intriguing, but they have not yet been tested by experimental manipulation. In another study, which did use an experimental approach, Shutler and Campbell [232], added yarrow (*Achillea millefolium*) to the nests of Tree Swallows (*Tachycineta bicolor*); this manipulation reduced the number of fleas in the nest by half, compared to control nests. However, the authors did not find that the use of green vegetation and the subsequent reduction in fleas had any effect on nestling survival or fledgling success. Gwinner *et al.* [233] manipulated green vegetation in European Starling (*Sturnus vulgaris*) nests and found no difference in the number of ectoparasites (mites, lice, fleas) between experimental and control nests; however, nestlings from nests with vegetation did have higher red blood cell counts and body masses than nestlings from nests without vegetation. The authors argued that the vegetation may stimulate the immune system of nestlings, which could ameliorate the detrimental effects of blood-feeding ectoparasites, even though it did not change parasite number, per se. This hypothesis has not been tested.

Yet another recent study, this one with Blue Tits (*Cyanistes caeruleus*), showed that in enlarged broods, nestling mass gain was positively affected by the addition of green vegetation [234]. However, there was no difference in fledgling body mass between chicks in nests with added vegetation versus control nests. In conclusion, these various studies reveal a link between green vegetation and decreased ectoparasite loads, and a link between vegetation and increased nestling condition. However, there is still no rigorous experimental evidence that fumigation of nests with green vegetation actually increases the fitness of birds by deterring parasites.

Heterospecific Cleaning

Birds can conceivably reduce ectoparasites using what Hart [24] referred to as "heterospecific cleaning". Both of the known cases involve nest maintenance. The most remarkable example was reported in a paper by Smith [235]. He observed that brood parasitic Giant Cowbirds (*Scaphidura oryzivora*) are tolerated by some nesting colonies of foster species, such as Yellow-rumped Caciques (*Cacicus cela*) and oropendolas (*Psarocolius wagleri, P. decumanus, Gymnostinops montezuma*). Smith [235] reported that the cost of brood parasitism was offset by the fact that the nestling cowbirds remove and consume parasitic botflies (*Philornis*) from the foster parents' offspring, thus enhancing the fledging success of the foster species. Selection for cowbird egg mim-

icry was relaxed under these conditions, explaining a higher frequency of non-mimetic eggs in colonies parasitized by flies. This "advantage of being parasitized" (by cowbirds) was reportedly lost in the case of cacique or oropendola colonies adjacent to large wasp nests, because the wasps deterred the flies from parasitizing bird nests. In such cases, cowbirds were not tolerated by the foster species and the frequency of mimetic eggs was higher. These interactions, which are among the most complex ever documented, need additional study and confirmation.

Gehlbach and Baldridge [236] reported another form of heterospecific cleaning. They documented higher growth rates of nestling Eastern Screech Owls (*Otus asio*) in nests with Blind Snakes (*Leptotyphlops dulcis*). Superficial scars on the snakes suggested they were transported to the nest by adult owls, yet not eaten. The authors argued that growth rates of young in nests containing snakes were higher because the snakes fed on parasitic larvae that are harmful to the nestlings. However, other factors could covary with snake presence and owlet growth, e.g., parental hunting ability. An experimental manipulation of snake presence is needed to test Gehlbach and Baldridge's [236] hypothesis.

Cleaning of one species of bird by another species, analogous to the cleaning symbioses of marine fishes, has also been suggested. Bowman and Billeb [237] speculated that the bizarre feeding behavior of "vampire" Sharp-beaked Ground Finches *(Geospiza difficilis)*, which puncture the pin-feathers of boobies to feed upon their blood, may have originated from finches feeding on the large, numerous hippoboscid flies that plague the boobies. This is an intriguing idea, but the authors were quick to point out that they did not actually observe finches feeding on hippoboscid flies. We are unaware of any other documented cases of cleaning interactions between different species of birds.

Nest Desertion

If all else fails, an ultimate strategy for dealing with nest parasites is simply to abandon the nest, rather than continuing to invest in offspring that may be doomed. Nest desertion in the face of high ectoparasite loads has been documented for many bird species [45, 212, 214, 238-245]. Duffy [242] showed that argasid ticks (*Ornithodoros amblus*) cause large-scale desertion of colonial seabird nesting colonies, which raises interesting questions. How often do adult birds desert because they are cutting their losses, versus simply escaping intolerable irritation? Because short-lived birds have fewer breeding seasons in which to reproduce, short-lived birds should be slower to abandon their nests than long-lived birds, all else being equal. Comparative and experimental studies are needed to investigate how life span affects the decision to desert nests, in the face of high ectoparasite load.

DISCUSSION

As we have tried to show, birds have an impressive array of possible defenses against ectoparasites. Different species of birds may use very different combinations of these defenses, but the extent to which this actually occurs is not known. Most work has focused on demonstrating what the various defenses are, and, in some cases, exactly how they function. A more complete understanding of how birds combat ectoparasites requires a broader perspective that considers how the different defenses interact, and the relationship of ectoparasite defense to the many other life history challenges birds face. The optimum strategy undoubtedly depends on various life history tradeoffs. Multiple defenses and how they interact are also important to document in order to better understand the nature of coevolutionary responses to host defense by the parasites themselves. The evolution of effective counterstrategies should be more difficult, all else being equal, if a host has more than one effective defense strategy.

Environmental constraints will also limit the defenses available to different species. Opportunities for sunning are more limited for birds that live in regions with an average of 300 days per year of rain (e.g., Seattle, Washington), compared to regions with an average of 300 days/year of sun (e.g., Salt Lake City, Utah). Redundant defenses may also be important in the face of environmental variability. Sunning and dusting may combat similar ectoparasites, but dusting is an option on a cloudy day, while sunning may be effective in habitats devoid of dust or loose dirt.

Composition of the ectoparasite community will also influence the defenses used by a given host. Ectoparasite species richness and abundance vary markedly among birds, even within single groups of ectoparasites [124]. Some species of tinamous (Tinamiidae) can be infested with a dozen species of lice, while ostriches (Struthionidae) have but one species [5, 63]. Defense strategies against a single species probably differ from those against a more diverse community. Consistent with this prediction, bird species known to host more species of lice appear to devote more time to maintenance behavior than birds with few species of lice [246]. This kind of relationship probably holds for other kinds of ectoparasites, as well.

Ectoparasite species richness can also vary within a single bird species. Brown and Wilson [247] compared the ectoparasite communities of House Sparrows in Europe and North America. They found that 34 of the 69 species of ectoparasites found on the European sparrows were "lost" when House Sparrows were introduced to North America. It would be interesting to test whether North American sparrows have lost certain defenses as a result of the reduction in the richness of their ectoparasite community.

Ectoparasite prevalence also varies across environments. A worldwide comparison of louse prevalence among 22 species of pigeons and doves (Columbidae) revealed a positive relationship between louse prevalence and ambient humidity [22]. For example, lice were found on fewer than 3% of birds in the Sonoran Desert of Arizona, whereas 92% and 100% of birds in Philippine and Peruvian rainforests, respectively, were infested. Moyer *et al.* [37] showed that pigeons and doves in arid habitats also have lower louse abundance than conspecifics in humid habitats. To confirm humidity as the causal agent, the authors experimentally manipulated the ambient humidity of captive feral Rock Pigeons. Louse infestations decreased greatly on birds kept at low humidity, compared with those at higher humidities.

The "arsenal" of defenses employed by a given bird species will also depend on adaptations that are not immediately

related to parasite control. Ornithologists have traditionally interpreted bill shape mainly as it relates to foraging [126, 248-250]. However, recent studies (e.g. Clayton *et al.* [125]; Fig. **4**) make it clear that selection for efficient preening can also play a role in the evolution of bill morphology. In environments with high ectoparasite pressure, selection for preening-efficient bills may be strong. Some species of birds have foraging ecology that precludes a maxillary overhang (e.g. woodpeckers, hummingbirds, oystercatchers (Fig. **2b**), skimmers, darters, and herons; Table **I**). These taxa are presumably under selective pressure to evolve other mechanisms for controlling ectoparasites.

Finally, ectoparasite defense may also vary in terms of investment in particular defenses, independent of other defenses. For example, preening birds cannot simultaneously forage or engage in courtship. Preening also reduces vigilance [251], increasing the risk of predation. Given these costs of preening, we predict that birds in areas of low ectoparasite pressure should spend less time preening than birds in areas of high ectoparasite pressure. This hypothesis would be easy to test simply by comparing preening rates of birds in different localities (e.g., Clayton and Cotgreave [105]). We expect that tradeoffs of this kind may be common for many of the different adaptations birds have for controlling ectoparasites.

CONCLUSION

Birds have a wide variety of defenses for combating their diverse communities of ectoparasites. In some cases there is overwhelming evidence that a particular trait is important in ectoparasite defense (e.g., preening). In other cases the purported defense has not been tested (e.g, dusting). In still other cases, recent evidence suggests that the purported defense may, in fact, have little to do with ectoparasite control (e.g. pectinate claws). Multiple defenses allow birds to target different types of ectoparasites and to defend themselves in the face of environmental variability. Birds should also modulate the use of particular defenses, as well as combinations of defenses, in response to the many life history demands they face.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publishers Web site along with the published article.

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APPENDIX B

DOES SUNLIGHT ENHANCE THE EFFECTIVENESS OF AVIAN PREENING FOR ECTOPARASITE CONTROL?

Abstract

Preening is a bird's first line of defense against harmful ectoparasites. Ectoparasites, in turn, have evolved adaptations for avoiding preening, such as hardened exoskeletons and escape behavior. Earlier work suggests that some groups of ectoparasites, such as feather lice, leave hiding places in feathers that are exposed to direct sunlight, making them more vulnerable to preening. It is therefore conceivable that birds may choose to preen in direct sunlight, assuming it improves the effectiveness of preening. Using Mourning doves and their feather lice, we tested two related hypotheses: 1) that birds with access to direct sunlight preen more often than birds in shade; and 2) that birds with access to direct sunlight are more effective at controlling their ectoparasites than birds in shade. To test these hypotheses, we conducted an experiment in which we manipulated both sunlight and preening ability. Our results provided no support for either hypothesis: birds given the opportunity to preen in direct sunlight did not preen significantly more often, nor more effectively, than birds in shade. Thus, the efficiency of preening for ectoparasite control appears to be independent of light intensity, at least in the case of Mourning doves and their feather lice.

Introduction

Birds have a variety of adaptations for combating ectoparasites, ranging from immunological responses (Owen et al., 2010) to morphological and behavioral defenses (Clayton et al., 2010). Preening behavior, which is usually the first line of defense, is effective against different groups of ectoparasites, including fleas, lice, flies, mites and ticks (Marshall, 1981). Preening has an energetic cost (Wooley and Owen, 1978), and it interferes with the ability of birds to engage in other behaviors, such as feeding or anti-

predator vigilance (Redpath, 1988). Despite these tradeoffs, the ubiquity of preening indicates that it plays a very important role, both for ectoparasite defense and other functions, such as straightening and cleaning of the feathers. Across taxa, birds spend an average of 9.2% of their time performing maintenance behavior, the large majority of which consists of preening (Cotgreave and Clayton, 1994). Other maintenance behaviors include scratching, bathing, dusting, sunning and anting (Simmons, 1964). For a recent review of the role of preening and other maintenance behaviors in ectoparasite control see Clayton et al. (2010).

Ectoparasites have a variety of morphological and behavioral adaptations for escaping host preening (Marshall, 1981). For example, lateral or dorso-ventral flattening of the body facilitates the rapid movement of parasites across feathers to escape preening. Most ectoparasites also have a thick cuticle that helps protect them from being crushed by the bill. Ectoparasites can also escape host preening by hiding; for example, some feather lice (Insecta: Phthiraptera) hide between the barbs of flight feathers, or they burrow into the downy regions of abdominal contour feathers (Bush et al., 2006). Recent work shows that cryptic coloration is yet another way in which feather lice can escape host preening (Bush et al., 2010). Species of host specific feather lice on light colored birds are lighter in color than species of lice on dark colored birds. Interestingly, species of lice confined to the head, which a bird can neither see, nor preen, are not cryptically colored.

The work by Bush et al. (2010) indicates that preening for ectoparasite control has an important visual component. The efficiency of preening for ectoparasite control may increase under bright light because most ectoparasites are negatively phototactic (Stenram, 1956; Marshall, 1981). Exposure to bright light causes some groups, such as

feather lice, to move out of interbarb spaces and across the feathers. The movement of lice from interbarb spaces increases their vulnerability to preening but it may also provide a visual stimulus for preening behavior, leading to increased preening when birds are in bright light (Caldwell et al., 2001). These observations yield two simple predictions. First, birds in bright light, such as direct sunlight, should preen more frequently than birds in shade. Second, birds given opportunities to preen in direct sunlight should eliminate more ectoparasites than birds kept in the shade.

To test these two hypotheses, we conducted an experiment with captive Mourning doves (*Zenaida macroura*) and their feather lice (*Columbicola baculoides*). Like all such lice, *C. baculoides* are permanent ectoparasites that spend their entire life cycle on the body of the host (Marshall, 1981). *Columbicola* spp. feed primarily on feathers and dead skin and decrease host mating success, thermoregulatory ability and survival (Clayton, 1990; Booth, 1993; Clayton et al. 1999). They will therefore exert selection on the host for efficient preening and other defenses (Clayton et al., 1999).

Methods

Forty-eight Mourning doves were captured using mist nets near Tucson, Arizona. They were transported to the University of Utah, Salt Lake City, where they were housed individually in 30 x 30 x 56 cm wire mesh cages in a windowless animal room with full spectrum fluorescent lighting. All birds were maintained on a 12 hr light/12 hr dark cycle and provided grain, grit and water *ad libitum*. These birds were used to culture lice for other experiments for over a year. They were then used in the experiment described herein, before being euthanized, as required by our IACUC committee. All research was conducted under IACUC protocol # 05-08009.

For 15 weeks prior to the start of the experiment, we reduced relative humidity in the animal room to a low level (< 30% rh) to kill 100% of the lice and eggs already present on the birds (Harbison et al., 2008). After the 15-week period, all birds were visually examined for 30-60 seconds for each of the following body regions: head, keel, back, rump, wings and tail (Clayton and Drown, 2001). No lice were found on any of the 48 birds, confirming the effectiveness of the low humidity procedure.

We used a 2 x 2 factorial design to investigate the effects of light exposure on preening and louse abundance. Birds were randomly assigned to a sun or shade treatment. Each group was further randomly subdivided into bitted (preening impaired) and not bitted (preening unimpaired) groups for a total of four treatments with 12 birds per treatment. Preening was impaired using plastic C-shaped bits that fit between the mandibles of the bird's bill. Bits create a 1-3 mm gap between the mandibles that disrupts the occlusion of the bill tips required for efficient preening, but without affecting feeding ability (Clayton et al., 2005). Birds in the unimpaired preening group were handled similarly to bitted birds. One week later each clean bird was "seeded" with 100 adult *C. baculoides* from a culture stock using methods described in Moyer et al. (2002). Throughout the experiment, the animal room was set at 24 ˚C and 50% rh - conditions at which *C. baculoides* thrive on captive Mourning doves (Malenke et al., 2011).

Two days after the 48 birds were seeded with lice, sunlight manipulation treatments were initiated. These treatments involved two-hour sessions each morning, during which all 48 cages were moved outdoors two hours after sunrise. Half of the cages were randomly assigned to the shade group, which had cotton fabric covering the top and side of the cage facing the early morning sun. The cages of birds assigned to the

sun group remained uncovered. The sessions took place in the early morning to prevent any risk of heat stress. Data loggers (HOBO® U12-001) placed directly on a subset of cages in the sun or shade recorded temperature and relative humidity. The experiment lasted 20 days (14 September 2007 – 3 October 2007). *Columbicola* take a mean (± SE) of 24.4 (± 0.3) days to mature to the adult stage from eggs (Martin, 1934). Therefore, the 20-day duration for the experiment was chosen because it allowed us to test for effects of treatment on the survival of a single cohort of lice.

The 48 cages were arranged randomly within a grid each morning. Each cage was in full view of one of two observers (JAHK or Sarah K. Huber). At the end of the 2-hour period cages were transferred back to the animal room. On overcast or rainy days (3 days in total) cages were not placed outdoors. During each session, the observers recorded preening and other behaviors using instantaneous scan sampling (Altmann, 1974). They recorded a total of 30 observations per bird per day with each observer making half of the observations for each bird.

At the end of the experiment all birds were euthanized, placed individually in plastic bags, and frozen. Later, each bird was thawed and subjected to a body washing procedure that accounts for 99% of the lice on a bird (Clayton and Drown, 2001). A two-way ANOVA was used to test for an effect of treatment (light exposure and bitting) on preening behavior and louse abundance. All values are presented as the mean (± SE). (Analyses were done in Prism v.5.0 (GraphPad software, Inc).

Results

Temperature and relative humidity varied predictably with treatment. Temperature in the sun, which was 20.79 (± 0.18)°C, was significantly greater than

temperature in the shade, which was 17.01 ± 0.18°C (Mann-Whitney, U = 89377, p < 0.0001). Over the course of the experiment, maximum temperature in the sun reached 29.69°C, and maximum temperature in the shade reached 24.85°C. Relative humidity was significantly lower in the sun, where it was 27.97 ± 0.35%, compared to the shade, where it was 34.65 ± 0.42% (U = 101105, p < 0.0001).

Neither sunlight, nor bitting, had a significant effect on the frequency of preening observed among groups (two-way ANOVA, light treatment, $F_{1, 44} = 1.22$, P = 0.28, bitting treatment, $F_{1, 44} = 0.002$, P = 0.96, light x bitting interaction, $F_{1, 44} = 0.08$, P = 0.78; Figure B.1). In contrast, there was a strong effect of bitting on adult louse abundance (bitting treatment, $F_{1, 44} = 18.46$, P < 0.0001). However, there was no effect of sunlight on adult louse abundance, nor any interaction between sunlight and bitting (light treatment, $F_{1, 44} = 0.07$, P = 0.79; light x bitting interaction, $F_{1, 44} = 0.04$, P = 0.84; Figure B.2).

The frequency of two other bird behaviors was also independent of treatment. There was no significant difference in the frequency of feeding between groups (light treatment, $F_{1, 44} = 1.54$, P = 0.22, bitting treatment, $F_{1, 44} = 1.29$, P = 0.26, light x bitting interaction, $F_{1, 44} = 0.65$, P = 0.42) nor was there a difference in the frequency of resting (light treatment, $F_{1, 44} = 2.78$, P = 0.10, bitting treatment, $F_{1, 44} = 0.001$, P = 0.96, light x bitting interaction, $F_{1, 44} = 2.63$, P = 0.11).

In contrast, there was an effect of sunlight on sunning, a behavior in which birds spread their wing and tail feathers while lying prone on the ground (light treatment, $F_{1, 44} = 13.02$, P < 0.001). Only birds with access to direct sunlight performed sunning behavior. However, sunning behavior was very uncommon, accounting for less than 1% of all behavior. There was no significant effect of bitting on sunning behavior, nor an

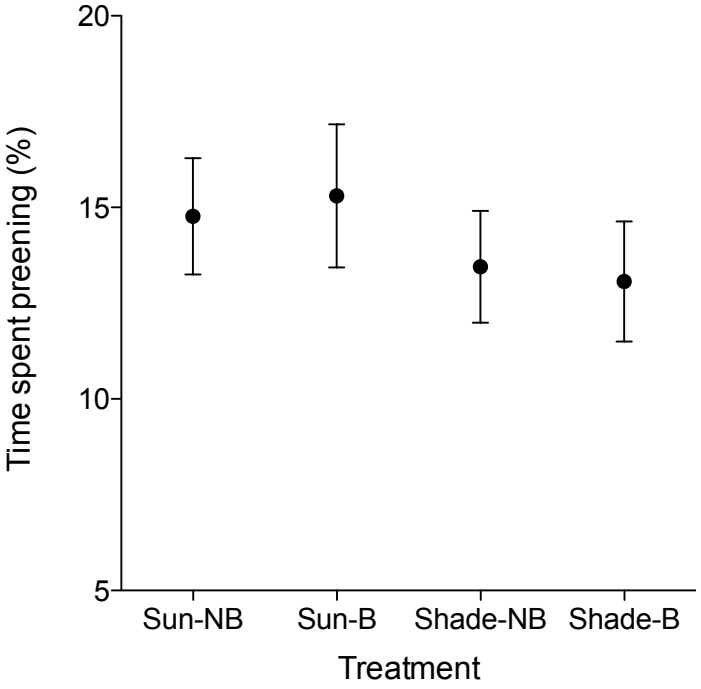

Figure B.1. Mean (± SE) percent time spent preening for each treatment group: exposed to sun (Sun), exposed to shade (Shade), not bitted (NB), and bitted (B).

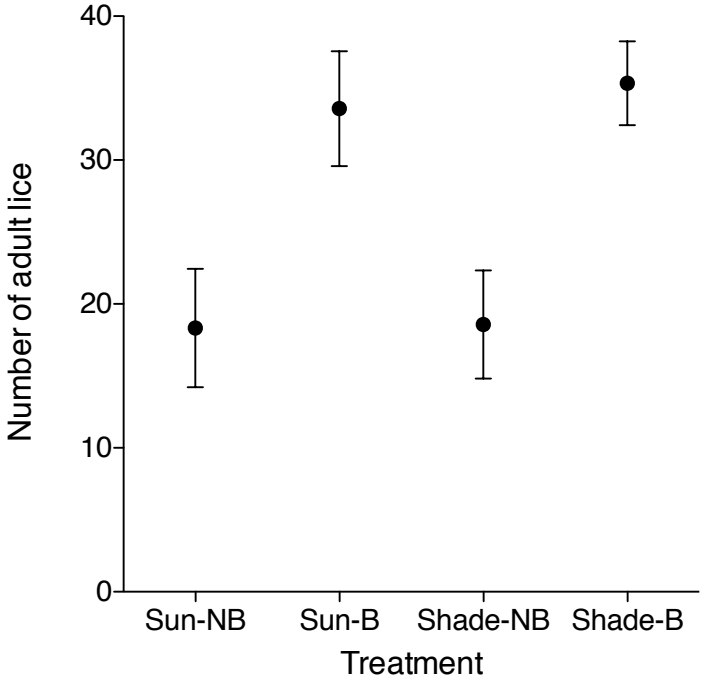

Figure B.2. Mean (± SE) number of adult lice at the end of the experiment: exposed to sun (Sun), exposed to shade (Shade), not bitted (NB), and bitted (B).

interaction between light exposure and bitting (bitting treatment, $F_{1, 44} = 2.46$, $P = 0.12$, light x bitting interaction, $F_{1, 44} = 2.457$, $P = 0.12$).

Discussion

The goal of this study was to explore the relationship between sunlight and preening for ectoparasite control. First, we tested the hypothesis that birds with an opportunity to preen in sunlight would preen more frequently than birds in shade. We also tested whether preening in sunlight is more effective at controlling ectoparasites than preening in the shade. To eliminate variation in parasite load at the start of the experiment, parasite-free birds were "seeded" with identical numbers of lice. We increased the probability of detecting effects on parasite load by infesting birds with 100 lice each - four fold the number found on wild Mourning doves in Utah (mean = 23.8, Malenke et al., 2011). At the end of the experiment, birds were euthanized and louse populations measured using a washing method that quantifies parasite load very accurately (Clayton and Drown, 2001). In summary, our experimental approach should have allowed us to detect even small treatment effects. By manipulating both access to sunlight and preening ability, the design of this experiment allowed us to test for direct and indirect effects of each factor on preening efficiency.

Sunlight may cause lice to move on feathers, increasing their vulnerability to host preening, and in doing so, providing an addition visual stimulus for preening behavior. Therefore, we predicted that birds in sunlight would increase their preening frequency. However, our results show that birds in sunlight do not, in fact, preen more than birds in shade (Figure B.1). This result suggests that sunlight is not a stimulus for preening behavior, at least in captive Mourning doves.

While the amount of time birds spent preening between treatments did not differ significantly, birds that preened in sunlight might still have been more effective at controlling their ectoparasites. However, our study further showed that birds with access to sunlight did not have significantly fewer lice at the end of the experiment than birds in shade (Figure B.2). Thus, sunlight does not appear to increase preening efficiency and thus, the ability of birds to control their ectoparasites.

In our study birds were exposed to direct sunlight or shade for two hours each day. Although this was only 17% of the diurnal phase of the experiment, it is more than the11-12% of time adult pigeons (*Columba livia)* spend preening in nature (Clayton, 1990), and more than the average 9.2% of time birds spend performing general maintenance behaviors (Cotgreave and Clayton, 1994). Of course, birds also spent time preening in the animal room. However, light intensity in the windowless animal room was clearly lower than in the light outdoors. Therefore, we expected birds to capitalize on conditions of direct sunlight outdoors, such that the two-hour period each day would have been sufficient to detect any difference in preening frequency and/or the effectiveness of preening between treatments.

Sunlight is also central to the ability of birds to combat ectoparasites with sunning behavior, during which birds often lie prone on the ground with their wing and tail feathers spread and their head feathers erect, facing directly into the sun (Simmons, 1986). Moyer and Wagenbach (1995) showed that the surface temperatures of feathers during bouts of simulated sunning are lethal to feather lice. However, these authors also found that birds perform sunning behavior only when the air temperature exceeds 29°C, which seldom occurred in our experiment. Sunning behavior was rare in our study,

comprising less than 1% of all recorded behaviors. Not surprisingly, only birds with access to direct sunlight engaged in sunning. Our experiment was purposefully carried out during relatively cool autumn weather, which avoided the potentially confounding effects of high temperature on host behavior and parasite survival. Future studies could use a similar experimental design, under conditions known to elicit more frequent sunning behavior, in order to test the effectiveness of sunning, per se, in controlling ectoparasites.

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