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Host-parasite interactions in Galapagos seabirds

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Host-Parasite Interactions in Galapagos Seabirds

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Dissertation abstract

Parasites exhibit a wide range of life history strategies that contribute to different dispersal abilities, host specialization, transmission modes, life-cycle complexity and population structure. Understanding dispersal rates in hosts and parasites is instrumental in defining the scale at which coevolution may be occurring. In order to better understand how and when parasites move between different hosts, I studied a seabird – Hippoboscid fly ectoparasite (and vector) – Haemosporidian parasite system in the Galapagos Islands. I began by describing the Haemosporidian parasites of Galapagos seabirds, discovering a *Plasmodium* species parasite in Galapagos Penguins (*Sphensicus mendiculus*), and a new clade of Hippoboscid-vectored parasites belonging to the subgenus *Haemoproteus* infecting frigatebirds (*Fregata* spp.) and gulls (*Creagrus furcatus*). Despite strong genetic differentiation between Galapagos frigatebirds and their conspecifics, we found no genetic differentiation in their *Haemoproteus* parasite. This led me hypothesize that the movement of the Haemosporidian parasite was facilitated by the movement of the Hippoboscid fly vector. In order to answer this question, I used a comparative population genetic study of Galapagos Great Frigatebirds (*F. minor*), Nazca Boobies (*Sula granti*), and their respective Hippoboscid fly parasites (*Olfersia spinifera*, *O. aenescens*) to better understand movement of flies at the geographic scale of the archipelago. I found high levels of gene flow in both fly species, despite marked differences in the degree of population genetic structure of their bird hosts. This suggests that host movement, (and therefore parasite movement), is not necessarily associated with true host dispersal, where dispersal is followed by successful

reproduction. Finally, I examined local (within island colony) transmission in the Great Frigatebird, *Haemoproteus iwa*, *Olfersia spinifera* system. I inferred movement, or host-switching, by analyzing host (frigatebird) microsatellite markers run on DNA amplified from the fly. Using the most variable microsatellite markers, we are able to identify host genotypes in bloodmeals that do not match the host from which the fly was collected. Flies that were not infected with *H. iwa* were more likely to have a bloodmeal that did not match the genotype of their host and female birds were the more likely recipients of host-switching flies.

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Note that seven chapters (I, II, V – IX) are manuscripts on which I am first author and for which I was primarily responsible. I am a co-author on one chapter (V) and chapter III contains two manuscipts, one of which I am a co-author on and the other I am co-first author.

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Chapter I: Haemosporidian Parasites: Impacts on avian hosts

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Haemosporidian parasites (order: Haemosporidia, phylum: Apicomplexa) are cosmopolitan intracellular protozoan parasites of birds, reptiles and mammals[30]. Haemosporidian parasites develop in two types of hosts, vertebrates and invertebrate vectors (Insecta: Diptera, blood-sucking dipterans); the dipteran is considered the definitive host as the site of sexual reproduction. Avian haemosporidia include parasites from three genera: *Plasmodium*, which is typically vectored by mosquitoes (Culicidae); *Haemoproteus*, which is primarily transmitted by biting midges (Ceratopogonidae) and louse flies (Hippoboscidae); and *Leucocytozoon*, which is vectored by blackflies (Simuliidae). Historically, *Plasmodium* has been considered potentially very pathogenic, and *Haemoproteus* relatively benign. In this chapter we will summarize studies relevant to these common perceptions and offer one detailed case study of an ongoing investigation of what is thought to be a recent arrival of *Plasmodium* in a naïve island population.

LIFE CYCLE OF HAEMOSPORIDIANS

The life cycle consists of several stages in both tissue and circulating blood cells of infected hosts. An infected vector feeds on vertebrate host blood, inoculating the host with sporozoites, giving rise to agamic stages (referred to as exoerythrocytic meronts or schizonts), which undergo asexual reproduction in fixed tissue in the host. This asexual division (often called merogony or schizogony) results in uninuclear merozoites. Another cycle of merogony occurs in the host blood cells in

Plasmodium, from which the parasite proceeds into the development of gametocytes; parasites in the genus *Haemoproteus* move quickly into the gametocyte stage in the blood. These cells produce macro- and microgametocytes, which are infective for the vectors. When an arthropod vector feeds on an infected bird, the change in carbon dioxide and oxygen concentrations initiate gametogenesis in the midgut of the vector, resulting in a sexual process called oogamy. Macrogametocytes produce macrogametes, microgametocytes produce microgametes, and fertilization occurs extracellularly. The zygote forms an elongated mobile ookinete, which penetrates the epithelial layer of the vector's midgut, where it develops into an oocyst. Sporozoites, the stage that is infective for the vertebrate hosts, are formed in the oocyst, and later move into the haemocoele of the vector, eventually penetrating the salivary glands. From there they can complete the infection cycle when the mosquito takes a second blood meal.

[Figure 1]

PATHOGENICITY

Pathogenicity of haemosporidian parasites is complicated and varied. Infection in bird hosts follows five main periods: prepatent, where parasite development occurs outside of the blood; acute, characterized by the appearance of parasites in the host blood and an increase in parasitemia; crisis, where parasitemia reaches a peak; and chronic/latent, a period of sharp decrease in parasitemia due to an immune response, following which parasitemia levels are then maintained at very low levels. Most research efforts aimed at understanding the effects of haemosporidia on host health examine hosts during the crisis and chronic stages, when we detect the

parasite in host erythrocytes by microscopy and amplify parasite DNA by polymerase chain reaction (PCR) from DNA extracted from host blood. Once infected, birds usually maintain parasites for years, and relapses tend to occur during host reproduction or other times of physiological stress.

Much of our understanding of the pathogenicty of haemosporidian parasites is based on laboratory experiments on domesticated birds (canaries, chickens, ducks, pigeons, turkeys) or on accounts from infections in birds housed in zoos. In a review of pathogenicity of haemosporidian parasites in birds, Bennett et al. found that 89% of published articles (5640 total) detailed mortality in domesticated birds while 6% and 5% pertained to mortality in zoo and wildlife populations respectively[6].

CAPTIVE POPULATIONS

Haemosporidian parasites (primarily *P. relictum* and *P. elongatum*) cause severe morbidity and mortality in penguin populations in zoos[6]. Most of the world's penguins are distributed near the poles, where haemosporidia are scarce. Therefore, many of the penguin species found in zoos have not evolved in regions that support populations of suitable vectors, resulting in naïve hosts, which in turn contributes to the severity of the infections. Many of the examples of mortality in zoos due to haemosporidia involve hosts challenged by parasites not found in their native distribution. Four Keas (*Nestor notabilis*) were captured in New Zealand and moved to the Malaysian National Zoo in 1964. Native Kea habitat in New Zealand was free of haemosporidia, but in captivity in Kuala Lumpur, where they were exposed to many blood-feeding vectors carrying local lineages of haemosporidia, all four died after three weeks in the new location due to infection by at least two *Plasmodium*

species[6]. Leucocytozoon species were found to be particularly pathogenic for birds in the orders Galliformes and Anseriformes (poultry and ducks)[6]

We cannot easily extrapolate findings from zoo or domesticated birds to wild hosts, partly due to the shared evolutionary history between hosts and their haemosporidian parasites in their native geographic distributions. Although the majority of haemosporidian parasites are not lethal in the wild, they may act as population modulators because they may reduce fitness, or reduce the competitive ability of infected individuals.

THE HAWAII EXAMPLE

We have learned a great deal about the impacts of haemosporidian parasites on wild populations in Hawaii. This example has been so instructive due to the very short evolutionary history that Hawaiian birds have with *Plasmodium*. Like haemosporidia in zoos, this situation is not entirely natural either; however, globalization, tourism and the pet trade contribute to a world where introduced diseases, like *Plasmodium* in Hawaii, are no longer unusual. Prior to 1826, there was no competent vector for *Plasmodium* in Hawaii. When the mosquito, *Culex quinquefaciatus*, was introduced to the islands, *Plasmodium relictum* spread through native and introduced bird populations, contributing to substantial mortality (65-100%) in several species of Hawaiian honeycreepers (Drepanididae). Intensive, long-term laboratory and field experiments have been conducted on Hawaiian avifauna providing us with a very complete understanding of the susceptibility of extant bird species to *Plasmodium*, the distribution (both across host species and in different habitats/elevation), and the prevalence (proportion of individuals infected) and intensity (proportion of cells

infected within an individual) of infections in birds and in vectors. Native species were more susceptible to *Plasmodium* than were introduced species and more likely to have detectable (by microscopy) infections during the non-breeding season[24]. Many surviving species, particularly the susceptible and consequently endangered ones, persist only above 1500 meters of elevation, where cooler temperatures prevent *Plasmodium* from effectively developing in mosquitoes. However, due to climate change and warmer temperatures, the prevalence of *Plasmodium* in Hawaiian forest birds sampled at 1900 meters has more than doubled in over a decade[11]. Some Hawaiian bird species appear to be coping; the Amakihi (*Hemignathus virens*), which exists in lowland areas where mosquitoes and *Plasmodium* are prevalent, showed no significant reduction in reproductive success (as measured by clutch size, hatching success, fledging mass, number of nestlings fledged, daily survival and minimum fledgling survival) while chronically infected with *Plasmodium relictum*[14]. These results are consistent with the hypothesis that offspring inherit genes for *Plasmodium* resistance from their infected parents that lead to increased survival, so it appears that the Amakihi is now a good reservoir for the parasite within the forest bird community. It remains unknown whether resistance will evolve in other species, since this requires both a growing population of resistant birds and heritable resistance to acute *Plasmodium* infection[14].

IMPACT IN LONG-TERM ASSOCIATIONS AND COMPARISON OF IMPACT ACROSS PARASITE GENERA

Haemosporidian parasites have been shown to impact hosts in situations where the hosts have presumably evolved with both the vectors and the parasites for far longer

than in the case of the Amakihi in Hawaii. Much of the research on fitness consequences of haemosporidian relies on correlative data in wild populations. While these studies are important in adding to our understanding of the impacts of these parasites, experimental manipulation may tease out the causal relationships involved. There are two main experimental approaches to understanding the impacts of haemosporidians on host fitness: brood size manipulation and medication experiments. By manipulating either the reproductive effort or by reducing natural parasite infection, experiments can reveal causal relationships. Both correlative and experimental studies that demonstrate a potential fitness cost to (and ones that show no effect of) haemosporidian parasites are summarized in Table 1.

[TABLE 1]

Overall, it is clear that haemosporidian parasites may have a significant impact on their hosts, both in situations where the parasite is recently introduced to naïve hosts and in situations where hosts have evolved with local lineages for a long period of time. Parasites, such as *Haemoproteus*, that have historically been considered relatively benign often impact their hosts significantly[15,17]. Studying the pathogenicity of haemosporidian parasites in nature is challenging due to a low probability of capturing a severely ill bird; weaker individuals are often not moving conspicuously or have been eliminated by predators. It is also important to keep in mind that these moderately to highly pathogenic parasites, that may often be handled by the host immune system, may become even more dangerous or lethal when the host is co-infected with another pathogen (or a second haemosporidian lineage/species). Already-infected hosts may have compromised immune systems

and be more susceptible to co-infection. More study, and particularly more long-term study, of the impacts of haemosporidian parasites on host survival and reproduction is needed to add to this growing area of research.

CASE STUDY: PLASMODIUM INFECTIONS IN GALAPAGOS PENGUINS

We have recently detected a *Plasmodium* species infecting Galapagos Penguins (*Spheniscus mediculus*)[15]. Penguins tend to be very susceptible to *Plasmodium* in captive situations[6], and Galapagos Penguins are considered endangered due to small population size and restricted geographical range. Galapagos Penguins exhibit low levels of genetic diversity[19] and very low variation in major histocompatibility complex (MHC) genes[7], both of which could contribute to the susceptibility of this population to infectious disease. The first task was to identify the parasite to the best of our ability and place it in a phylogenetic context to begin to understand the potential for pathogencity.

The two *Plasmodium* species that cause severe morbidity and mortality in captive penguin populations are *P. relictum* and *P. elongatum*, belonging to the subgenera Haemamoeba and Huffia respectively. We detected (by PCR and subsequent DNA sequencing) *Plasmodium* in 5% of 362 penguins tested[15]. Our phylogenetic analysis placed the parasite sequences within *Plasmodium* close to a *P. elongatum* sequence and other sequences belonging to the Huffia subgenus. The 19 positive penguins were widely distributed across 9 sites of 5 islands in the Galapagos. Genetic analyses demonstrate that these penguins may move long distances[19], and we know that *Plasmodium* infections may be long-lasting, suggesting that the locations of infected penguins may tells us little about where (and when) the infections were

contracted. Galapagos Penguins are severely affected by El Niño; population sizes are reduced by as much as 50% during an El Niño year[31]. Penguins (n=94) sampled before the most recent El Niño all tested negative for Haemosporidian parasites[18], suggesting that the population has not yet had to face the combined challenges of *Plasmodium* infection and the stressful environmental conditions of an El Niño year.

ONGOING WORK IN GALAPAGOS

Having identified what we think is a recently-arrived *Plasmodium* species infecting the Galapagos Penguin, we have embarked on an extensive plan to determine: (1) whether it is infecting other species; (2) to identify the reservoir population; and (3) to identify the arthropod vector. We will discuss each of these in turn.

If the *Plasmodium* infecting the penguins is a recent arrival, we have grave concerns that a number of Galapagos endemic species may also be susceptible due to their long isolation without exposure. We have sampled a very large number of passerine birds along the coastlines where penguins congregate, knowing that infections must be originating where the parasite is completing its life cycle within a resident population, and where the penguins are being bitten by the same arthropod vectors as the reservoir host.

We believe that the infections in penguins are not being sustained by a penguin-mosquito-penguin cycle, as this would require successful completion of the life cycle to the gametocyte stage within penguins. We have never seen the gametocyte stage in blood smears from Galapagos Penguins, suggesting to us that the transmission cycle is through a reservoir species as yet unidentified, and that when infected mosquitoes bite Galapagos Penguins, the penguins become dead-end hosts.

A good reservoir species would be one that is benign in both directions, with the parasite having little impact on the host and the host little impact on the parasite, the sort of relationship of mutual tolerance that permits both host and parasite to survive and reproduce in optimal fashion. This well-equilibrated relationship is more likely to have evolved in a host-parasite relationship of long duration. Since *Plasmodium* appears to be a recent arrival to Galapagos, this cannot characterize its relationship with any of the endemic lineages that have been there for hundreds of thousands or millions of years without exposure.

To date, we have found no evidence for *Plasmodium* infections in any other endemic birds of hundreds tested to date including passerines of several finch species, yellow warblers, and mockingbirds, and including other nonpasserines such as the cormorants that share the penguins' range. We have not yet covered the entire coastal range of the penguins, however, and know that somewhere they are contracting infections that have successfully cycled through a bird host, and so we will continue to search. We have no evidence that the parasite has yet infected other endemic species.

In our search for the reservoir species we have focused initially on the only two introduced bird species currently residing on the islands, Smooth-Billed Anis (*Crotophaga ani*) and Cattle Egrets (*Bubulcus ibis*). Anis were first introduced by humans during the 1960's in the hope that they would reduce the tick burden on cattle[23], and while they are slated for eradication, they still occur in large numbers on several islands of the archipelago. In a sample of 60 anis collected from the island of Santa Cruz, where they are considered an invasive species, we found three that tested

positive for haemosporidian blood parasites by PCR, and those three amplifications sequenced as identical to the *Plasmodium* sequence from penguins. It is thus possible that the exotic ani is the reservoir species, or at least one of a number of competent reservoirs. We will also test the Cattle Egrets that were first documented in the 1960's and that are suspected to also have been introduced, although the situation by which they arrived is uncertain. In either case, both species occur in large numbers on the South American mainland (and Cattle Egrets throughout the world) where their ancestors have had long histories of exposure to haemosporidian parasites.

Finally, we will continue our work to identify the arthropod vector. Since *Plasmodium* is typically vectored by mosquitoes (Culicidae), we are trapping and testing mosquitoes of the three species occurring on the Galapagos Islands, the Black Salt-Marsh Mosquito *Aedes taeniorhynchus*, the Southern House Mosquito *Culex quinquefasciatus*, and the Yellow Fever Mosquito *Aedes aegypti*. The Yellow Fever Mosquito is thought to be strongly specific to biting humans, and so is not considered a likely candidate, but we will test it as new host-parasite relationships may arise more commonly on islands where population densities of preferred hosts are sometimes very low. The Black Salt-Water Mosquito arrived naturally to the archipelago some 200,000 years ago[4] and is common throughout the archipelago on coastlines and other moist habitats and is capable of breeding in brackish water. The Southern House Mosquito is known to be the vector for *Plasmodium relictum* in Hawaii and has been established in Galapagos since the 1980's[33]. Unlike *A. taeniorhynchus, C. quinquefasciatus* requires fresh water to reproduce and so will be restricted in Galapagos to the small number of areas with regular standing fresh

water, which are also the sites inhabited by humans. For all three species, our tests will involve trapping blood-meal-searching females and identifying the source of blood meals through molecular techniques, and then testing for the presence of *Plasmodium* by PCR for any species identified as feeding on birds. The final identification of vector status will require dissection of salivary glands for microscopic examination for the *Plasmodium* sporozooite stage.

CAN IT BE ERADICATED?

We think there are circumstances under which this pathogen may be eradicated from the archipelago before any of the Galapagos endemic birds suffer the same sad fate as the Hawaiian honeycreepers. These conditions are:

(1) That the vector is identified as the Southern House Mosquito *Culex quinquefasciatus*. We think this is the most likely candidate because of its role as vector for *Plasmodium relictum* in Hawaii. Because of its requirement of freshwater, its distribution is severely restricted in Galapagos compared to that of *A. taeniorhynchus*[5]. With this level of localization, and with the historical success of malarial eradication through mosquito control, (we are optimistic that this may be accomplished. *Because C. quinquefasciatus* is a recent arrival[33], it is eligible for eradication, unlike any native species.

(2) That the reservoir species is identified as either the Smooth-Billed Ani (*Crotophaga ani)* or the Cattle Egret (*Bubulcus ibis*) or both. Given their status as introduced species, either or both of these species are eligible for eradication.

(3) That no endemic species has become a reservoir.

(4) That the Galapagos National Park, that oversees all management efforts on the islands, will undertake the eradication of Culex, Anis, Cattle Egrets, or all three, in a historic attempt to divert a conservation crisis. The history of success in eradications in Galapagos of introduced birds (rock pigeons) and especially the destructive feral pigs, donkeys, and goats[8] suggests to us that the willingness and commitment necessary for a program of this magnitude exists on the islands.

CONCLUSIONS

Studies of avian haemosporidian parasites have been increasing in number, partly due to the ease of testing for these parasites using molecular techniques. We have learned much from situations like Hawaii, and from the growing body of evidence that, in many cases, haemosporidian parasites may have detrimental effects on reproduction and survival. The majority of the research on the impacts of haemosporidians is still correlative, and we need more experimental manipulation to investigate causal relationships between all the variables, particularly when correlations between some measure of haemosporidian infection and fitness can be explained in multiple ways. Additionally, relationships between fitness measures and parasitism may not be linear. A recent paper shows that for Blue Tits (*Cyanistes caeruleus*) infected with *Haemoproteus*, maximum survival was found at intermediate levels of parasitism[28]. A significant negative quadratic effect was found between host survival and parasite intensity, suggesting that high parasite intensities are detrimental to the host, but that there are also costs of controlling the parasites at low levels. Therefore, there may be a cost to being resistant (at least via actively mounting an immune response to

suppress infection). More attention ought to be given to the possibility of non-linear relationships between fitness costs of parasitism and haemosporidian infection. Additionally, we encourage more work in experimental infection as well as exploring new frontiers in haemosporidian research involving multiple infections (with either two species of haemosporidia or haemosporidia(ns) and another parasite/pathogen).

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FIGURE LEGENDS

Figure 1: General schematic of the haemosporidian lifecycle

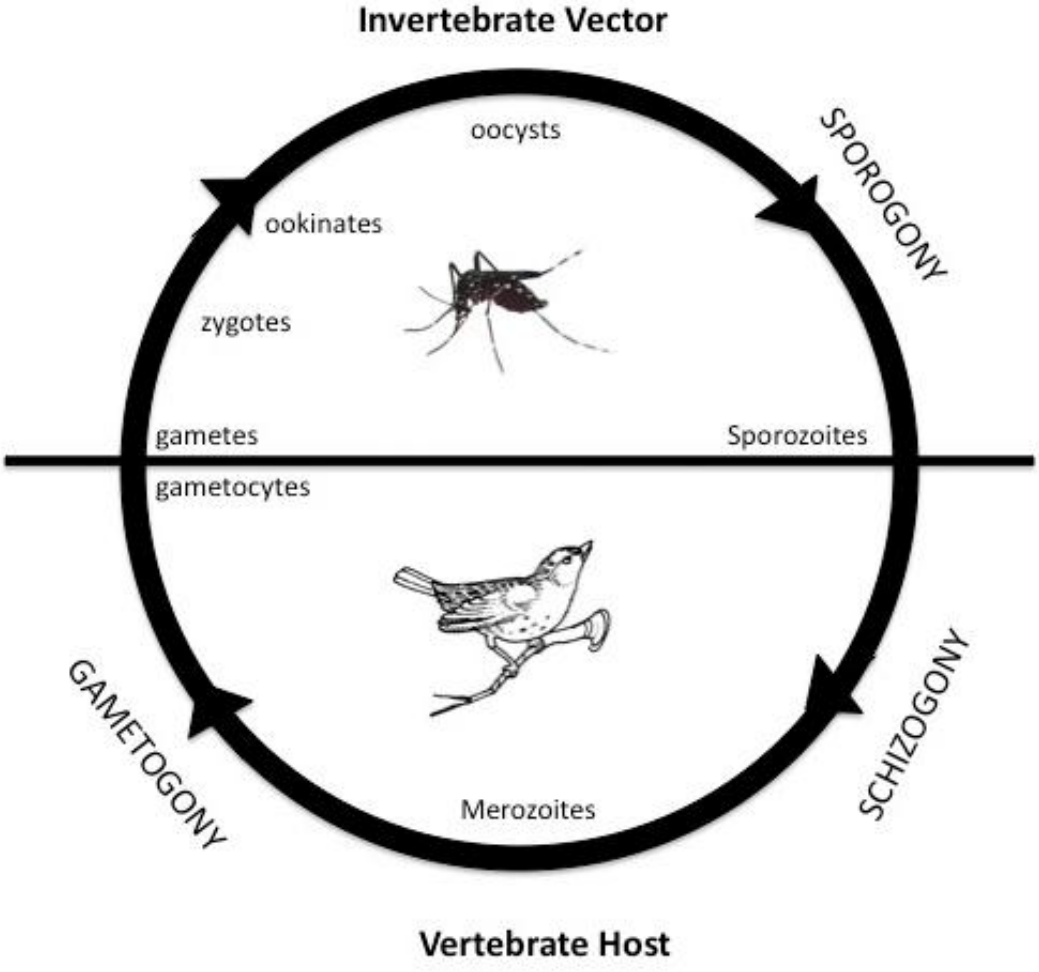
Table 1: Summarized results of studies measuring impacts of haemosporidian parasites, separated into those showing negative impacts of haemosporidian infection and those that do not demonstrate an effect.

Table 1: Summarized results of studies measuring impacts of haemosporidian parasites, separated into those showing negative impacts of haemosporidian infection and those that do not demonstrate an effect.

EXAMPLES SHOWING AN EFFECT OF PARASITISM				
Parasite	**Host**	**Impact Measured**	**Result**	**Reference**
Plasmodium	Great Reed Warblers (*Acrocephalus arundinaceus*)	Primary (experimental) infection on previously uninfected juveniles vs. chronic infections in adults	Naïve birds developed higher parasitemias; mortality rates in experimentally infected juveniles was high, although not all attributed just to haemosporidian infection (co-infection with Isospora)	29
		Co-infection of naïve birds with two Plasmodium lineages	Strong positive correlation between parasitemias for both lineages	29
Plasmodium, *Haemoproteus*, *Leucocytozoon*	Great Tit (*Parus major*)	Body condition and plasma protein levels	Negatively affected by *Leucocytozoon* and *Plasmodium*	16
		Red blood cell glutathione peroxidase activity	Higher activity in birds infected with *Leucocytozoon* and *Plasmodium*	16
		Reproduction (egg weight)	Females that laid heavier eggs had higher probabilities of being infected by Plasmodium when feeding nestlings	16
Haemoproteus	Great Tit	Egg laying, hatching	Delayed	1
Haemoproteus	American Kestrel (*Falco sparverius*)	Female condition	Poorer during incubation	6
		Female return rate	Lower for birds with higher intensity infections	6
Leucocytozoon, *Plasmodium*	White-Crowned Sparrow (*Zonotrichia leucophrys oriantha*)	Song behavior	Infected birds responded less to playback; song consistency affected	9
Haemoproteus	Red-Wing Blackbird (*Agelaius phoeniceus*)	Dominance	Uninfected individuals tended to be more dominant	27
Plasmodium	Great Tit	Brood size manipulation	Males attending enlarged broods had significantly higher prevalence	17
Leucocytozoon, *Haemoproteus*, *Hepatozoon*	Blue Tit (*Parus caeruleus*)	Brood size manipulation	Females caring for enlarged broods had higher intensity infections	7
Haemoproteus	Blue Tit	Brood size manipulation	Poor nestling condition resulting from enlarged broods	22

			positively correlated with reduced long-term ability to control haemosporidian infections.	
Haemoproteus	Blue Tit	Medication experiment	Higher fledging success in broods of medicated females	14
Haemoproteus	House Martin (*Delichon urbica*)	Medication experiment	Larger clutches in broods of medicated females, higher hatching and fledging success	13
EXAMPLES SHOWING NO EFFECT OF PARASITISM				
Plasmodium	Hawaiian Thrushes (*Myadestes* spp.)	Serological response, mortality, subsequent re-infection	Minor transient infections followed by immunity when re-challenged with the parasite	2
Haemoproteus	Lesser Kestrels (*Falco naumanni*)	Clutch size, Adult survival	No effect	24
Leucocytozoon	Mallard (*Anas platyrhynchos*), American Black Duck (*Anas rubripes*)	Duckling growth	No negative effect	21
Haemoproteus	Red-Bellied Woodpecker (*Melanerpes carolinus*)	Female condition, male and female survival	No effect; however, survival only measured by year-to-year survival over a one year period	20
Haemoproteus	Great Tit	Brood size manipulation	No effect of enlarged broods on parasite intensity	10

FIGURE 1


Invertebrate Vector
oocysts
SPOROGONY
ookinates
zygotes
gametes
Sporozoites
gametocytes
GAMETOGONY
SCHIZOGONY
Merozoites
Vertebrate Host

Chapter II: Plasmodium blood parasite found in endangered Galapagos penguins (*Spheniscus mendiculus*)

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Abstract: This is the first report of a *Plasmodium* blood parasite found in the Galapagos Archipelago. Phylogenetic analyses place this parasite, recovered from endangered Galapagos penguins (*Spheniscus mendiculus*), within the genus *Plasmodium*, and suggest a close relationship to some of the most dangerous lineages of *Plasmodium* that have been known to cause severe mortality and morbidity in captive penguin populations. Infectious disease is an increasingly important cause of global species extinctions, and extinctions due to avian pox and avian malaria (*Plasmodium relictum*) have been well documented in Hawaiian avifauna. *Plasmodium* blood parasites had not been detected in Galapagos birds until now, despite previous microscopic and molecular screening of many of the species, including the Galapagos penguin. While penguin populations now appear healthy, it is unclear whether this parasite will have an obvious impact on their survival and reproduction, particularly during El Niño events, which cause stress due to reduced food availability. It is possible that this parasite arrived with or shortly after the recent arrival of an introduced mosquito, *Culex quinquefasciatus*, known elsewhere as a competent vector of *Plasmodium* blood parasites.

Introduction

The Galapagos Islands are located on the equator approximately1000 km west of continental Ecuador. Humans have inhabited the archipelago for 200 years, and much of the original biodiversity remains intact, with only 5% species loss (Gibbs et

al., 1999). Due to isolation and high endemism, there is concern regarding the introduction of diseases. Island populations are often more susceptible to introduced pathogens, as they have historically been exposed to fewer pathogens than mainland populations (e.g., Fromont et al., 2001). Introduced pathogens, primarily avian pox (Avipoxvirus) and avian malaria (*Plasmodium relictum*) are a likely cause of major population declines and extinctions in Hawaiian avifauna (van Riper et al., 1986, 2002). Ongoing disease monitoring is an essential part of conservation efforts in Galapagos (Parker et al., 2006) to prevent extinction due to introduced diseases, increasingly recognized as causes of global wildlife extinctions worldwide (Smith et al., 2006). Here we report a blood parasite in the genus *Plasmodium* found in the endemic Galapagos penguin, which could threaten the health of penguins and other bird species. *Plasmodium*, *Haemoproteus* and *Leucocytozoan* (suborder Haemosporina, phylum: Apicomplexa) are related genera of vector-borne protozoan blood parasites commonly found throughout reptiles, birds and mammals. Some *Plasmodium* species are pathogenic and cause disease in wild and captive animals. While *Haemoproteus* parasites appear to have fewer detrimental effects on hosts, some fitness reductions have been documented (e.g., Allander, 1997). Avian malaria, the disease in birds caused by some parasites in the genus *Plasmodium*, causes considerable morbidity and mortality in outdoor penguin exhibits in zoos, where pathogenic species are identified as *P. relictum* and *P. elongatum* (e.g., Fleischman et al., 1968; Stoskopf and Beier, 1979). While many of the world's penguins are distributed in the Antarctic region, some species breed at lower latitudes in temperate environments, where they may naturally encounter these parasites (Graczyk et al.,

1995). There are concerns regarding Plasmodium parasites in penguins, due, in part, to the acute infections found in captive populations (Fleischman et al., 1968; Stoskopf and Beier, 1979; Fix et al., 1988; Cranfield et al., 1994). There are few reports of blood parasites in wild penguins (e.g., Jones and Shellam, 1999), but the potential for *Plasmodium* to cause disease in endangered or geographically isolated bird populations is grounds for concern and monitoring (Jones and Shellam, 1999; Miller et al., 2001).

The Galapagos penguin (*Spheniscus mendiculus*) is endemic to the Galapagos Islands and classified as Endangered (BirdLife International, 2008) due to small population size and restricted geographical range. El Niño events reduce populations of the Galapagos penguin by as much as 50% (Vargas et al., 2006), as warmer waters disrupt upwelling of nutrient-rich cold water that supports the marine ecosystem. The current population of Galapagos penguins is approximately 1500 individuals (Jiménez-Uzcátegui and Vargas, 2008). Galapagos penguins exhibit low levels of genetic diversity (Nims et al., 2008) and very low variation in major histocompatibility complex (MHC) genes (Bollmer et al., 2007), which could contribute to the susceptibility of the population to infectious disease. Overall, the Galapagos penguin population appears healthy, based on surveys of hematology, serum chemistry and serology (Travis et al., 2006). No intra-erythrocytic blood parasites were found in microscopic screens of blood smears (Travis et al., 2006). Galapagos penguins (n = 94) sampled in 1996 were tested for *Plasmodium* using a molecular screening technique (polymerase chain reaction (PCR)), and no penguins tested positive (Miller et al., 2001).

Materials and methods

Sample collection

Between August 2003 and March 2005, a total of 401 samples were collected from 362 Galapagos penguins captured during four field seasons at 29 sites from seven islands of the Galapagos Archipelago (Table 1, Fig. 1). Due to close proximity and small area, the three Mariela islands are considered here as one Island (Marielas). All tested penguins were marked with microchips (PIT tags) for identification and assessment of survivorship in subsequent field seasons. Details on sample collection, processing and analysis, can be found in Travis et al. (2006).

Molecular screening

DNA was extracted from blood using a standard phenol–chloroform extraction protocol (Sambrook et al., 1989), and PCR was used to amplify a region of the parasite mitochondrial cytochrome *b* gene. Positive and negative controls were always used and test samples were only run with other Galapagos penguin samples to avoid interspecific contamination. A subset of positive samples were re-amplified to confirm that the first test showed true positive and not contamination. Primers included an initial outer reaction (DW2 and DW4) followed by an internal re-amplification (HaemoR and DW1; Perkins and Schall, 2002). Reaction conditions for DW2 and DW4 were identical to Perkins and Schall (2002) except for the addition of an initial dwell at 94° for 2 min and an annealing temperature of 55° instead of 60° C. Touchdown reaction conditions for HaemoR and DW1 are: initial dwell at 94° for 2 min, followed by 20 cycles of 94° for 30 s, 54° for 30 s (decreasing by 0.5° each cycle) and 72° for 90 s. The program then has 25 cycles of 94° for 30 s, 44.5° for 30 s

and 72° for 90 s and a final extension for 15 min. PCR reactions were performed using Takara Ex taq polymerase (Takara Bio Inc.). One microliter of stock DNA was used in the initial reaction, and 0.5 μ of product from the initial reaction was used as a template for the internal re-amplification reaction. Approximately 600 base pairs of double-stranded sequence were obtained on an Applied Biosystems 3100 DNA Analyzer at the University of Missouri – St. Louis.

Phylogenetic analysis

Sequences were edited in Seqman 4.0, added to a larger dataset containing additional cytochrome *b* sequence data obtained from GenBank (Appendix A, electronic supplement), and aligned using BioEdit (Version 7.0.9.0). Using parameters estimated from the data, the HKY85+I+C (Hasegawa et al., 1985) model of nucleotide substitution was used to reconstruct a maximum clade credibility phylogeny (BEAST, 10,000 trees; Drummond and Rambaut, 2007) with maximum likelihood branch lengths (PAUP 4.0) and in a ML bootstrap analysis (500 pseudoreplicates) (Treefinder, Jobb, 2008). BEAST initiates a pre-burn-in to stabilize likelihood values, after which it begins sampling. Parameters in BEAST allow for mutation rate heterogeneity among branches of the phylogeny, in which any biases due to disproportionately long branches are reduced (relaxed clock: uncorrelated lognormal). Priors for the model were optimized by the program using the Yule tree option. Unlike coalescent approaches in which only some lineages are assumed to leave descendants, the Yule tree option assumes that such lineages have already been pruned (Drummond and Rambaut, 2007). The likelihood stationarity of sampled trees

was determined graphically via a log-likelihood frequency histogram in Tracer (v1.4; Rambaut and Drummond, 2007).

Results

The PCR screen identified 19 (5%) of 362 penguins as positives for *Plasmodium*. The prevalence of the parasite in the four field seasons ranged from 3% to 7% and did not show a tendency to increase from 2003 to 2005 (Table 1). Most positive penguins were found on northern and western Isabela as well as on Santiago and Bartolomé Islands (Fig. 1). Two penguins that tested positive in the first sampling season were in good health conditions when recaptured in subsequent sampling seasons after seven and 12 months, respectively, and still tested positive (Table 1). Based on molecular sexing data, the 19 positive penguins consisted of 14 adult males and 5 females, three of which were juveniles. Because screening primers amplify both *Haemoproteus* and *Plasmodium* parasites, DNA sequencing and phylogenetic analysis were used for identification. Phylogenetic analyses place all but one of the Galapagos penguin parasite sequences within a large clade containing all *Plasmodium* parasites (Fig. 2). Galapagos penguin *Plasmodium* sequences are distinct from any other available sequences, and form their own evolutionary unit or clade. Their position within the larger *Plasmodium* clade is near a *P. elongatum* sequence and sequences belonging to the subgenus, *P. huffia*, which includes *P. elongatum*, although this placement does not have strong support. While nearly all of the sequences from this parasite can be unequivocally assigned to the genus *Plasmodium*, one parasite sequence from a Galapagos penguin sequence clustered with *Haemoproteus* (*Haemoproteus* 11).

Discussion

This is the first time a blood parasite in the genus *Plasmodium* has been identified in a Galapagos bird. Our phylogenetic inference places this parasite within the genus *Plasmodium* and sister to a clade containing *P. elongatum*, a parasite known to cause avian malaria in penguins and *P. huffia*, the subgenus that contains *P. elongatum* (Fleischman et al., 1968; Cranfield et al., 1994). There is strong support for the inclusion of the blood parasite in Galapagos penguins within *Plasmodium*, but weaker support for a particular sister clade within *Plasmodium*. More sequence data from additional genes and longer sequences could help resolve some of these relationships. One sequence recovered from penguins clustered with *Haemoproteus* sequences, and, to our knowledge, is the first reported *Haemoproteus* parasite in a penguin.

Despite the lack of resolution within *Plasmodium* and uncertainty of the exact sister taxa, we recommend that management strategies consider that this *Plasmodium* is closely related to a species that causes acute avian malaria in captive penguins. Penguins appear susceptible to serious infection by *P. relictum* and *P. elongatum*, and the Galapagos penguin is likely immunologically naïve since it evolved in an isolated island system. Immunological naïveté has been implicated as an important factor in the loss of Hawaiian avifauna due to introduced avian malaria and avian pox (van Riper et al., 1986). If this parasite is recently introduced, it could have disastrous consequences due to the lack of immunity or past exposure that would protect populations from serious infection. Our only evidence suggesting it might not be a

pathogenic parasite under benign circumstances is that none of the penguins testing positive in our study showed any clinical indication of illness (see Travis et al., 2006). The only arthropod present in Galapagos that is known to be a competent vector for *Plasmodium* elsewhere is the mosquito *Culex quinquefasciatus*, first reported in 1989 and well established by 2003 (Whiteman et al., 2005). Miller et al. (2001) suggest there could be a connection between the introduction of *C. quinquefasciatus* and the disappearance of resident penguins on the north shore of the human-inhabited island of Santa Cruz. The other bird-biting mosquito in the archipelago is a native, brackish-water mosquito, *Ochlerotatus taeniorhynchus* (sometimes called *Aedes taeniorhynchus*). Extensive sampling of mosquito populations around penguin colonies is necessary in order to further characterize this parasite, identify its vector, and develop an appropriate management strategy. The *Plasmodium* sequences recovered from Galapagos penguins belong to one phylogenetic lineage whose members are genetically similar, which also suggests a recent arrival with insufficient time for further differentiation. A final piece of evidence suggesting this is a newly introduced parasite is that Miller et al. (2001) found no infected penguins of 96 tested in 1996 using a similar PCR protocol. Based on our prevalence estimates, we would have detected approximately five positive birds with a similar sample size.

The 19 positive penguins were widely distributed across 9 sites of five islands in the Archipelago (Fig. 1). No *Plasmodium* parasites were detected in sites of the southern portion of the penguin distribution and this may be related to the low sample sizes (1 from Santa Cruz, 3 from Floreana and 12 from Puerto Villamil in southern Isabela) and low densities of penguins that limited capturing success. It is possible

that the parasite will soon become widespread along the whole distributional range of the penguin population as recent genetic evidence suggests that the penguins may move long distances (Nims et al., 2008), at least during some part of their lives, and infections can be long-lasting. This also suggests that locations of infected penguins in this study may tell us little about where those infections were contracted.

Given that Galapagos penguins are severely affected by El Niño events, the additional stress caused by an infection with *Plasmodium* could lead to a more serious population decline. Stress has been demonstrated to be positively correlated with *Plasmodium* prevalence (Richner et al., 1995). In experimentally enlarged broods, male Great tits (*Parus major*) increased their feeding effort by 50% and had significantly higher prevalence of *Plasmodium* parasites than males attending control broods (Richner et al., 1995). The last El Niño event occurred in 1997–1998, and based on Miller et al.'s (2001) 1996 sampling and findings, we have no evidence to believe that *Plasmodium* parasites were infecting penguins during this stressful El Niño event. Therefore, the combined effects of *Plasmodium* parasitism and stronger (and more stressful) El Niño events in light of future climate change scenarios could place this endangered population at an even greater risk of extinction (see Vargas et al., 2007). We recommend immediate action to identify the vector for this parasite, and continued monitoring of penguin populations as well as other bird populations at risk of infection.

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Table 1. Number of samples and Plasmodium prevalence in 362 PIT-tagged penguins studied during four field seasons in the Galapagos Islands between 2003 and 2005.

	Field seasons				
Island	August 2003	March 2004	August 2004	February-March 2005	Total
Isabela	36 (4)	80 (4)	65 (3)	61 (2)	242 (13)
Marielas	12	20	25	37 (2)	94 (2)
Fernandina	26 (1)	7	1	6	40 (1)

Number in parenthesis indicates number testing positive for *Plasmodium*.

Bartolomé				14 (2)	14 (2)
Santiago				7 (3)	7 (3)
Floreana				3	3
Santa Cruz				1	1
Total samples	74 (5)	107 (4)	91 (3)	129 (9)	401 (21)
Prevalence %[(+)]	7	4	3	7	5
Penguins [(-)] recaptured	0	7	8	22	37
Penguins [(+)] recaptured	0	1	1	0	2[a]
Total penguins	74	99	82	107	362

[(+)] *Plasmodium* positive

[(-)] *Plasmodium* negative

[a] Tested positive for the first time in August 2003

Figure legends

Figure 1: Spatial distribution of *Plasmodium* in the Galapagos Islands in 2003-2005 based on GPS locations. Red dots indicate locations of positive samples. Green squares are sampling sites. Black dots show distribution of the penguin population during the annual census in September 2005. Penguins are not resident breeders on Santa Cruz. Numbers in parentheses show prevalence of *Plasmodium* at each site (number of positive samples/number of total samples).

Figure 2: Maximum clade credibility phylogenetic hypothesis of haemosporidian parasites based on mitochondrial *cytochrome b*. ML bootstrap values appear above nodes and Bayesian posterior probabilities appear below nodes. Parasite lineages are detailed in Appendix A and listed in the order within the phylogeny (top to bottom).

Figure 1:

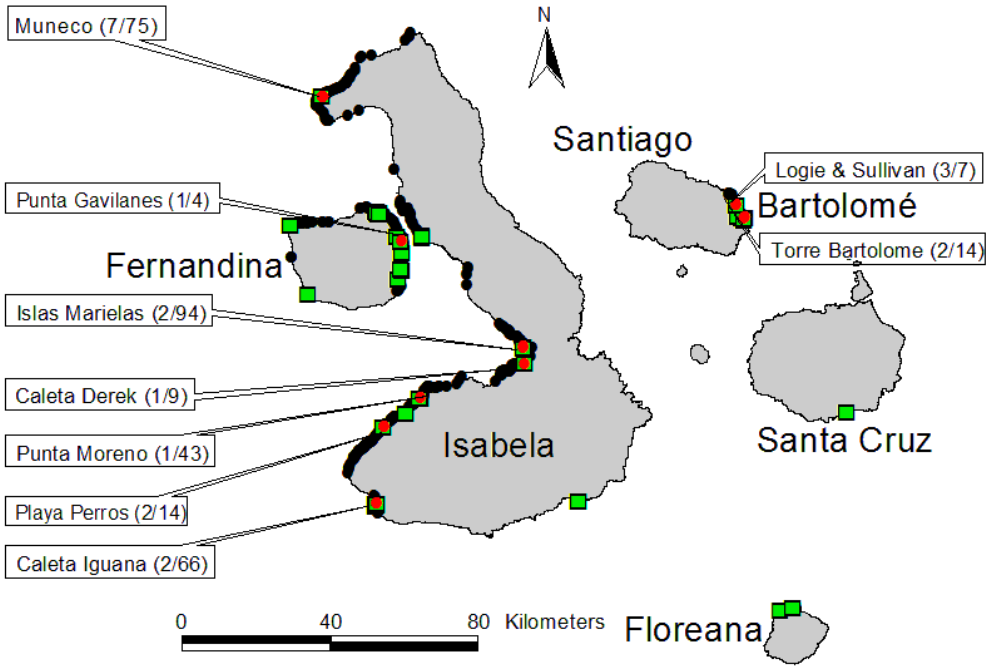
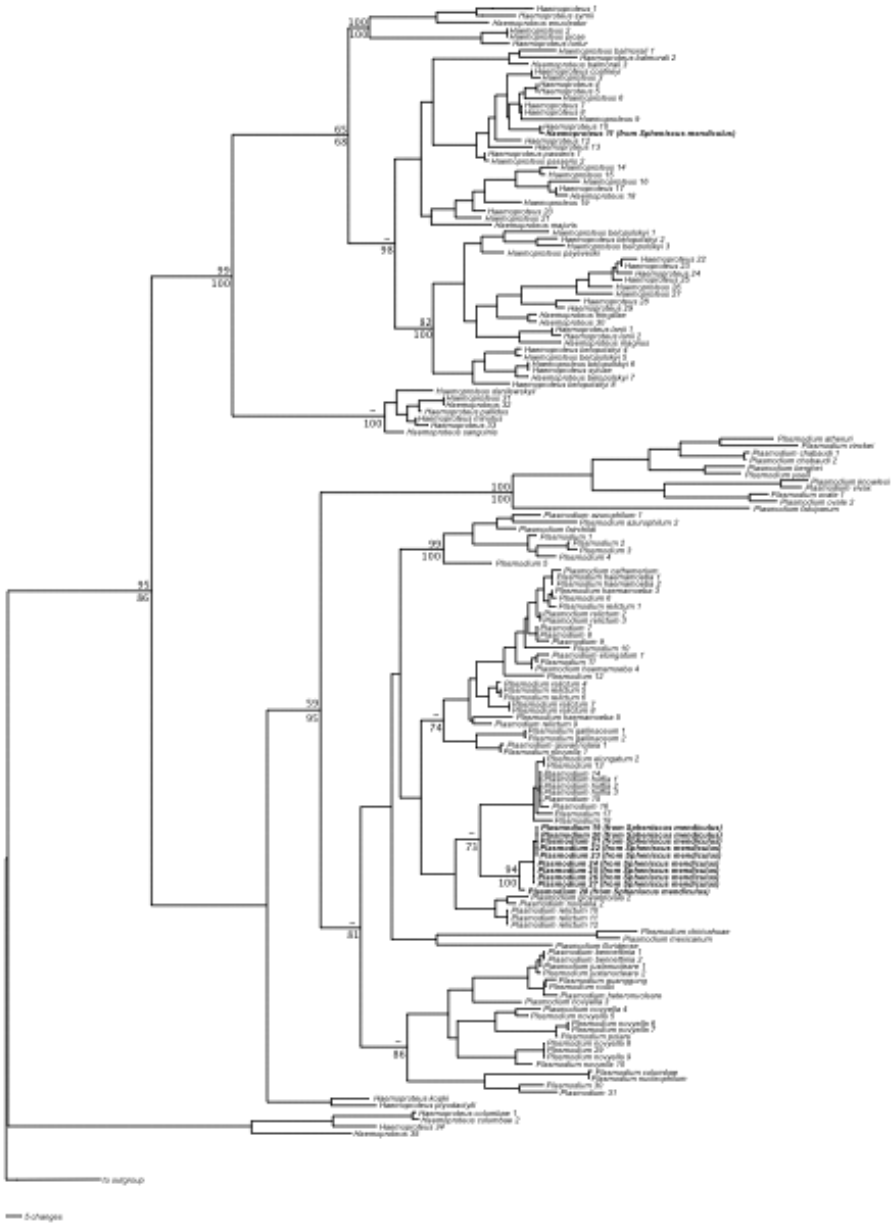

Muneco (7/75)
Punta Gavilanes (1/4)
Fernandina
Islas Marielas (2/94)
Caleta Derek (1/9)
Punta Moreno (1/43)
Playa Perros (2/14)
Caleta Iguana (2/66)
N
Santiago
Logie & Sullivan (3/7)
Bartolomé
Torre Bartolome (2/14)
Isabela
Santa Cruz
0
40
80 Kilometers
Floreana

Figure 2:



Appendix A: Samples included in analyses

Sequence name	**Accession number**	**Citation**
Haemoproteus 1	GQ395631	
Haemoproteus syrnii	DQ451424	Martinsen et al. 2006
Haemoproteus enucleator	DQ659592	Beadell et al. 2006
Haemoproteus 2	GQ395666	
Haemoproteus picae	EU254552	Martinsen et al. 2008
Haemoproteus turtur	DQ451425	Martinsen et al. 2006
Haemoproteus balmorali 1	DQ630007	Hellgren et al. 2007
Haemoproteus balmorali 2	DQ630008	Hellgren et al. 2007
Haemoproteus balmorali 3	DQ630014	Hellgren et al. 2007
Haemoproteus coatneyi	EU254550	Martinsen et al. 2008
Haemoproteus 3	GQ395671	
Haemoproteus 4	GQ395637	
Haemoproteus 5	GQ395667	
Haemoproteus 6	GQ395661	
Haemoproteus 7	GQ395651	
Haemoproteus 8	GQ395658	
Haemoproteus 9	GQ395683	
Haemoproteus 10	GQ395678	
Haemoproteus 11	GQ395686	
Haemoproteus 12	GQ395633	
Haemoproteus 13	GQ395655	
Haemoproteus passeris 1	EU254554	Martinsen et al. 2008
Haemoproteus passeris 2	DQ451422	Martinsen et al. 2006
Haemoproteus 14	GQ395632	
Haemoproteus 15	GQ395690	
Haemoproteus 16	GQ395672	
Haemoproteus 17	GQ395673	
Haemoproteus 18	GQ395674	
Haemoproteus 19	GQ395676	

Haemoproteus 20	GQ395663	
Haemoproteus 21	GQ395649	
Haemoproteus majoris	AY099045	Perkins and Schall 2002
Haemoproteus belopolskyi 1	DQ451408	Martinsen et al. 2006
Haemoproteus belopolskyi 2	DQ451427	Martinsen et al. 2006
Haemoproteus belopolskyi 3	DQ451428	Martinsen et al. 2006
Haemoproteus payeveski	DQ451430	Martinsen et al. 2006
Haemoproteus 22	GQ395634	
Haemoprotues 23	GQ395638	
Haemoprotues 24	GQ395652	
Haemoprotues 25	GQ395647	
Haemoprotues 26	GQ395653	
Haemoprotues 27	GQ395635	
Haemoprotues 28	GQ395659	
Haemoprotues 29	GQ395689	
Haemoproteus fringillae	EU254558	Martinsen et al. 2008
Haemoprotues 30	GQ395668	
Haemoproteus lanii 1	DQ630011	Hellgren et al. 2007
Haemoproteus lanii 2	DQ630012	Hellgren et al. 2007
Haemoprotues magnus	DQ451426	Martinsen et al. 2006
Haemoprotues belopolskyi 4	DQ451412	Martinsen et al. 2006
Haemoprotues belopolskyi 5	DQ630006	Hellgren et al. 2007
Haemoprotues belopolskyi 6	DQ451416	Martinsen et al. 2006
Haemoproteus sylvae	AY099040	Perkins and Schall 2002
Haemoprotues belopolskyi 7	DQ451417	Martinsen et al. 2006
Haemoprotues belopolskyi 8	DQ451419	Martinsen et al. 2006
Haemoproteus danilewskyii	DQ451411	Martinsen et al. 2006
Haemoproteus 31	GQ395656	
Haemoproteus 32	GQ395664	
Haemoproteus pallidus	DQ630005	Hellgren et al. 2007
Haemoproteus minutus	DQ630013	Hellgren et al. 2007
Haemoproteus 33	GQ395665	

Haemoproteus sanguinis	AY178904	Zhu et al. unpublished
Plasmodium atheruri	AY099054	Perkins and Schall 2002
Plasmodium vinckei	AY099052	Perkins and Schall 2002
Plasmodium chabaudi 1	AY099050	Perkins and Schall 2002
Plasmodium chabaudi 2	EF011167	Martinsen et al. 2007
Plasmodium berghei	AY099049	Perkins and Schall 2002
Plasmodium yoelii	AY099051	Perkins and Schall 2002
Plasmodium knowlesi	AF069621	Escalante et al. 1998
Plasmodium vivax	AF069619	Escalante et al. 1998
Plasmodium ovale 1	AB182497	Win et al. 2004
Plasmodium ovale 2	AF069625	Escalante et al. 1998
Plasmodium falciparum	AY588280	Musset et al. 2006
Plasmodium azurophilum 1	AY099055	Perkins and Schall 2002
Plasmodium azurophilum 2	AY099058	Perkins and Schall 2002
Plasmodium fairchildi	AY099056	Perkins and Schall 2002
Plasmodium 1	DQ337362	Austin and Perkins 2006
Plasmodium 2	DQ337363	Austin and Perkins 2006
Plasmodium 3	DQ337365	Austin and Perkins 2006
Plasmodium 4	DQ337364	Austin and Perkins 2006
Plasmodium 5	DQ337361	Austin and Perkins 2006
Plasmodium cathermerium	AY377128	Wiersch et al. 2005
Plasmodium haemamoeba 1	EF011180	Martinsen et al. 2007
Plasmodium haemamoeba 2	EF011192	Martinsen et al. 2007
Plasmodium haemamoeba 3	EF011183	Martinsen et al. 2007
Plasmodium 6	GQ395679	
Plasmodium relictum 1	DQ659543	Beadell et al. 2006
Plasmodium relictum 2	DQ659544	Beadell et al. 2006
Plasmodium relictum 3	DQ659540	Beadell et al. 2006
Plasmodium 7	GQ395657	
Plasmodium 8	GQ395669	
Plasmodium 9	GQ395691	
Plasmodium 10	GQ395681	

Plasmodium elongatum 1	AF069611	Escalante et al. 1998
Plasmodium 11	GQ395688	
Plasmodium haemamoeba 4	EF011185	Martinsen et al. 2007
Plasmodium 12	GQ395677	
Plasmodium relictum 4	DQ659553	Beadell et al. 2006
Plasmodium relictum 5	DQ659555	Beadell et al. 2006
Plasmodium relictum 6	DQ659556	Beadell et al. 2006
Plasmodium relictum 7	DQ659563	Beadell et al. 2006
Plasmodium relictum 8	EF011193	Martinsen et al. 2007
Plasmodium haemamoeba 5	EF011194	Martinsen et al. 2007
Plasmodium relictum 9	EU254538	Martinsen et al. 2008
Plasmodium gallinaceum 1	AY099029	Perkins and Schall 2002
Plasmodium gallinaceum 2	EU254535	Martinsen et al. 2008
Plasmodium giovannolaia 1	EF011187	Martinsen et al. 2007
Plasmodium novyella 1	EF011172	Martinsen et al. 2007
Plasmodium elongatum 2	DQ659588	Beadell et al. 2006
Plasmodium 13	GQ395650	
Plasmodium 14	GQ395648	
Plasmdoium huffia 1	EF011168	Martinsen et al. 2007
Plasmdoium huffia 2	EF011178	Martinsen et al. 2007
Plasmdoium huffia 3	EF011175	Martinsen et al. 2007
Plasmodium 15	GQ395654	
Plasmodium 16	GQ395680	
Plasmodium 17	GQ395675	
Plasmodium 18	GQ395682	
Plasmodium 19	GQ395640	
Plasmodium 20	GQ395645	
Plasmodium 21	GQ395643	
Plasmodium 22	GQ395644	
Plasmodium 23	GQ395684	
Plasmodium 24	GQ395641	
Plasmodium 25	GQ395685	

Plasmodium 26	GQ395642	
Plasmodium 27	GQ395646	
Plasmodium 28	GQ395687	
Plasmodium gionvannolaia 2	EF011188	Martinsen et al. 2007
Plasmodium novyella 2	EF011181	Martinsen et al. 2007
Plasmodium relictum 10	EU254536	Martinsen et al. 2008
Plasmodium relictum 11	AY099032	Perkins and Schall 2002
Plasmodium relictum 12	DQ659589	Beadell et al. 2006
Plasmodium chiricahuae	AY099061	Perkins and Schall 2002
Plasmodium mexicanum	AY099060	Perkins and Schall 2002
Plasmodium floridense	AY099059	Perkins and Schall 2002
Plasmodium bennettinia 1	EF011197	Martinsen et al. 2007
Plasmodium bennettinia 2	EF011198	Martinsen et al. 2007
Plasmodium juxtanucleare 1	AB302893	Murata et al. 2008
Plasmodium juxtanucleare 2	DQ017964	Elisei et al. unpublished
Plasmodium guanggong	AY178903	Zhu et al. unpublished
Plasmodium rouxi	AY178904	Zhu et al. unpublished
Plasmodium heteronuceare	AY178902	Zhu et al. unpublished
Plasmodium novyella 3	EF011177	Martinsen et al. 2007
Plasmodium novyella 4	EF011184	Martinsen et al. 2007
Plasmodium novyella 5	EF011190	Martinsen et al. 2007
Plasmodium novyella 6	EF011171	Martinsen et al. 2007
Plasmodium novyella 7	EF011182	Martinsen et al. 2007
Plasmodium polare	DQ659590	Beadell et al. 2006
Plasmodium novyella 8	EF011189	Martinsen et al. 2007
Plasmodium 29	GQ395670	
Plasmodium novyella 9	EF011170	Martinsen et al. 2007
Plasmodium novyella 10	EF011174	Martinsen et al. 2007
Plasmodium columbae	AF069613	Escalante et al. 1998
Plasmodium nucleophilum	AF254962	Bensch et al. 2000
Plasmodium 30	GQ395660	
Plasmodium 31	GQ395662	

Haemoproteus Kopki	AY099062	Perkins and Schall 2002
Haemoproteus ptyodactylii	AY099057	Perkins and Schall 2002
Haemoproteus columbae 1	AF069613	Escalante et al. 1998
Haemoproteus columbae 2	EU254548	Martinsen et al. 2008
Haemoproteus 34	GQ395636	
Haemoproteus 35	GQ395639	
Outgroup: Leucocytozoon lovati	AB183550	Sato et al. 2007
Outgroup: Leucocytozoon squamatus	DQ451432	Martinsen et al. 2006
Outgroup: Leucocytozoon macleani	DQ676825	Sehgal et al. 2006
Outgroup: Leucocytozoon schoutedeni	DQ676823	Sehgal et al. 2006
Outgroup: Leucocytozoon simondi	AY099064	Perkins and Schall 2002
Outgroup: Leucocytozoon majoris	AY099045	Perkins and Schall 2002
Outgroup: Leucocytozoon gentili	DQ451434	Martinsen et al. 2006
Outgroup: Leucocytozoon dubreuli	AY099063	Perkins and Schall 2002

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Chapter III: New Haemosporidian parasite descriptions

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and

II. Levin, I.I., Valkiunas, G., Iezhova, T.A., O'Brien, S.L. and P.G. Parker. Novel *Haemoproteus* species (Haemosporida: Haemoproteidae) from the Swallow-Tailed Gull (Lariidae), with remarks on the host range of Hippoboscid-transmitted avian hemoproteids. *In press*, Journal of Parasitology.

I. **ABSTRACT:** *Haemoproteus (Haemoproteus) multipigmentatus* n. sp. (Haemosporida, Haemoproteidae) was found in the endemic Galapagos dove *Zenaida galapagoensis*. It is described based on the morphology of its blood stages and segments of the mitochondrial cytochrome *b* gene, which can be used for molecular identification and diagnosis of this species. *Haemoproteus multipigmentatus* can be readily distinguished from all species of hemoproteids of the subgenus *Haemoproteus*, primarily due to numerous (approximately 40 on average) small pigment granules in its mature gametocytes. Illustrations of blood stages of the new species are given, and phylogenetic analysis identifies DNA lineages closely related to this parasite, which is prevalent in the Galapagos dove and also has been recorded in other species of Columbiformes in Mexico, Guatemala, and Peru, so seems to be widespread in countries with warm climates in the New World. Cytochrome *b* lineages of *H. multipigmentatus* cluster with hippoboscid transmitted lineages of *Haemoproteus columbae*. The same lineages of *H. multipigmentatus* were recorded in thoraxes of the hippoboscid fly *Microlynchia galapagoensis*, which likely is a natural vector of this parasite in Galapagos. This study shows that more discussion among researchers is needed in order to clearly establish the sequence length and number of

genes used for identification of hemosporidian parasites at different taxonomic levels**.** Because different primers might amplify different parasites if they have a better match during a simultaneous infection, it is important that researchers standardize the genetic marker of choice for molecular typing of hemosporidian species. We point to the need of using both morphology and gene markers in studies of hemosporidian parasites, particularly in wildlife.

INTRODUCTION

During an ongoing study on the distribution and evolutionary biology of pathogens in Galapagos (Padilla et al., 2004; Parker et al., 2006; Santiago-Alarcon et al., 2008; Levin et al., 2009; Santiago-Alarcon et al., 2009), blood samples and hippoboscid flies (Hippoboscidae) were collected from the endemic Galapagos dove *Zenaida galapagoensis* and other columbiform birds in the New World between 2002 and 2009. One previously undescribed species of *Haemoproteus* (Haemosporida, Haemoproteidae) was found during this study. This parasite is described here using data on the morphology of its blood stages, and partial sequences of the mitochondrial cytochrome *b* (cyt *b*) gene. We also identify a probable vector of this hemoproteid in the Galapagos archipelago and generalize available information about its distribution and avian host range. Some problems of molecular identification and diagnostics of hemosporidian parasites using partial DNA sequences are also discussed.

MATERIAL AND METHODS

Collection of blood samples and hippoboscid flies

In all, 443 blood samples were collected from doves and pigeons in North and South America and the West Indies between 2002 and 2009. The birds were caught

with mist nets and hand nets. We collected 170 blood samples from Galapagos doves on 10 islands of the Galapagos archipelago (Santiago, Santa Cruz, Santa Fe, Española, San Cristobal, Genovesa, Marchena, Fernandina, Darwin, and Wolf). Blood samples were also obtained from 17 species of columbiform birds belonging to 7 genera in the United States (2 samples), Mexico (7), Caribbean islands (10), Venezuela (126), Peru (29), Uruguay (2), Ecuador (73), and Guatemala (10). Samples from Ecuador (Galapagos and the mainland), Peru, and USA were collected by the authors. Samples from other localities were provided to us by colleagues (for details about study sites and investigated bird species, see Santiago-Alarcon et al., 2009). For a description of the new species of parasite, samples from 10 Galapagos doves and 3 continental species of Columbiformes were used; these samples were selected based on the availability and quality of blood smears for morphological work, and on the close similarity among Galapagos and mainland parasite lineages, as identified by Santiago-Alarcon et al. (2009).

Blood was taken by puncturing the brachial vein; all birds were then released with none of the individuals being recaptured. Approximately 50 µl of whole blood was drawn from each bird for subsequent molecular analysis. The samples were preserved in lysis buffer (Longmire et al., 1988), and then held at ambient temperature in the field and later at –20 C in the laboratory.

Blood smears were collected only from Galapagos doves. Blood films were air-dried within 5-10 sec after their preparation; they were fixed in absolute methanol in the field and then stained with Quick Field's stain (2002-2008 samples) and in Giemsa (2009 samples) in the laboratory. Blood films were examined for 10-15 min

at low magnification (×400) and then at least 100 fields were studied at high magnification (×1,000). Detailed protocols of preparation, fixation, staining, and microscopic examination of blood films are described by Valkiūnas, Iezhova, Križanauskienė et al. (2008). Intensity of infection was estimated as a percentage by actual counting of the number of parasites per 1,000 red blood cells or per 10,000 red blood cells if infections were light, i.e., <0.1%, as recommended by Godfrey et al. (1987). To determine possible presence of simultaneous infections with other hemosporidian parasites in the type material of new species, the entire blood films from hapantotype and parahapantotype series were examined microscopically at low magnification.

Hippoboscid flies *Microlynchia galapagoensis* were collected by hand during bird manipulation, directly from the plumage of Galapagos doves. The insects were stored in 95% alcohol in the field and later at 4 C in the laboratory until DNA extraction and subsequent testing by PCR. Seven individual flies were used in this study.

Morphological analysis

An Olympus BX61 light microscope equipped with Olympus DP70 digital camera and imaging software AnalySIS FIVE was used to examine slides, prepare illustrations, and to take measurements. The morphometric features studied (Table I) are those defined by Valkiūnas (2005). Morphology of new species was compared with the type and voucher specimens of hemoproteids of the subgenus *Haemoproteus* from their type vertebrate hosts belonging to the Columbidae: *Haemoproteus columbae* (host is Rock dove *Columba livia*, accession nos. 2905.87, 47723 NS,

47724 NS in Collection of Institute of Ecology, Nature Research Centre, thereafter CNRC), *Haemoproteus sacharovi* (Mourning dove *Zenaida macroura*, nos. 45236A, 45236B, 103700 in Queensland museum, Queensland, Australia, and no. 47739 in the CNRC), *Haemoproteus turtur* (Turtle dove *Streptopelia turtur*, no. 1315.87 in the CNRC), and *Haemoproteus palumbis* (Woodpigeon *Columba palumbus*, 969, 970 in the Natural History Museum, London, UK and no. 2067.87 in the CNRC). Student's *t*-test for independent samples was used to determine statistical significance between mean linear parameters. A *P*-value of 0.05 or less was considered significant.

DNA extraction, PCR amplification, and sequencing

Infections were determined by microscopic examination of blood smears and by PCR amplification of parasite gene sequences. DNA was extracted by phenol-chloroform method followed by dialysis in 1X TNE_2 (Sambrook and Russell, 2001). Published primers and protocols from Waldenström et al. (2004) were used to amplify a fragment of the parasites' mitochondrial cytochrome *b* (cyt *b*) gene. PCR products were cleaned directly using Antarctic phosphatase and Exonuclease I (# M0289S and # M0293S respectively, New England Bio Labs, Inc., Ipswich, Massachusetts). We used an ABI 3100 microcapillary genetic analyzer to sequence DNA products. Sequences were edited in 4Peaks v1.7.2 (2005, http://mekentosj.com/science/4peaks/) and aligned by eye in Se-Al v2.0a11 (1996–2002, http://tree.bio.ed.ac.uk/software/seal/). New sequences were deposited in GenBank™ (accession numbers: GU296210 – GU296227).

In the laboratory, thoraxes of 7 hippoboscid flies *M. galapagoensis* were carefully severed from heads and abdomens. Each thorax was used individually for

DNA extraction; we used a Qiagen DNEasy Blood and Tissue DNA extraction kit (Qiagen, Valencia, California). The standard protocol was followed, however DNA was eluted in half as much buffer due to assumed low concentrations of any parasite DNA. Protocols for PCR amplification and sequencing were as described above.

To ensure that the positive PCR results from insects were DNA from sporozoites and not from some undigested parasite infected blood cells that might have persisted in the vector digestive system as remnants of blood meal, thoraxes of all insects were tested for bird mitochondrial cyt *b* gene with primers and protocols used in Ngo and Kramer (2003). Galapagos dove mitochondrial DNA was used as a positive control to identify and compare bird DNA amplified from insect thoraxes.

Phylogenetic analysis

The phylogenetic history of *Haemoproteus multipigmentatus* and related hemosporidian parasites was reconstructed by using sequence information from our former studies and GenBank™ for the mitochondrial cytochrome *b* gene. Because GenBank™ contains information about numerous incorrectly identified species of hemosporidians (see Valkiūnas, Atkinson et al., 2008), we used mainly sequences of positively identified avian parasites (for examples of linking parasite lineages with their morphospecies, see Križanauskienė et al., 2006; Sehgal et al., 2006; Hellgren et al., 2007; Valkiūnas et al., 2007; Palinauskas et al., 2007; Martinsen et al., 2008; Valkiūnas, Atkinson et al., 2008; Valkiūnas, Iezhova, Loiseau et al., 2008; Svensson and Ricklefs, 2009; Valkiūnas et al., 2009; Iezhova et al., 2010).

Phylogenetic hypotheses were constructed using the program Mr. Bayes v3.1.2 (Huelsenbeck and Ronquist, 2001). We performed 3 independent runs, with 4

chains in each run for a total of 3 million generations, sampling every 100 generations. First 15,000 trees were discarded as the "burn-in" periods. In total, 15,000 trees from each run were used to build our majority-rule consensus tree. For the analyses, we used a GTR+I+Γ model of molecular evolution with shape parameter $\alpha = 0.45$, and proportion of invariable sites Pinvar = 0.34 as calculated from the data using Mr. Bayes v3.1.2.

The sequence divergence between the different lineages was calculated with the use of a Jukes-Cantor model of substitution, with all substitution weighted equally, implemented in the program MEGA 3.1 (Kumar et al., 2004).

RESULTS

Description

***Haemoproteus (Haemoproteus) multipigmentatus* n. sp.**

(Figs. 1-16, Table I)

Young gametocytes (Figs. 1- 2): Develop in mature erythrocytes. Earliest forms seen anywhere in infected erythrocytes, but more frequently recorded lateral to erythrocyte nuclei; markedly variable in shape. With development, gametocytes extend along nuclei of erythrocyte, touching neither nuclei nor envelope of erythrocytes (Fig. 1). Pigment granules small (< 0.5 μm), black, and frequently grouped (Fig. 2). A few roundish, light-violet small volutin granules usually present. Outline of growing gametocytes wavy (Fig. 1), irregular (Fig. 2), or slightly ameboid. Influence of young gametocytes on infected erythrocytes usually not pronounced.

Macrogametocytes (Figs. 3- 12): Extend along nuclei of erythrocytes; elongate slender bodies with wavy, irregular, or slightly ameboid outline. Cytoplasm

blue, homogeneous in appearance, usually possesses small (< 0.5 μm), light-violet volutin granules and few vacuoles; small (< 1 μm in diameter) azurophilic granule frequently seen (Fig. 8). Growing gametocytes, with length exceeding length of erythrocyte nuclei (Figs. 3-5), have no permanent position in relation to nuclei or envelope of erythrocytes; usually lying free in cytoplasm, not touching either nuclei or envelope of erythrocytes (Fig. 3); also seen touching nucleus or envelope of erythrocytes (Figs. 4, 5), but usually not both these cellular structures at this stage of development. Advanced gametocytes do not displace or only slightly displace nuclei of erythrocytes; usually in touch with both erythrocyte nuclei and envelope, filling erythrocytes up to their poles (Fig. 6). Mature gametocytes extend around nuclei of erythrocytes, enclosing them with their ends, but do not encircle nuclei completely (Figs. 7, 8); they usually push nuclei with their middle part to envelope of erythrocytes (Fig. 7) and finally occupy nearly entire cytoplasmic space in host cells (Fig. 9). In advanced gametocytes, 2 clear unfilled spaces appear between ends of gametocytes and nuclei of erythrocytes (Figs. 7, 8), giving gametocytes horn-like appearance, and disappearing as parasite matures (Figs. 9-11). Fully-grown gametocytes closely associated with nuclei and envelope of erythrocytes, filling erythrocytes up to their poles (Figs. 9-11). Parasite nucleus small (Table I), variable in form, frequently irregular in shape, median or submedian in position (Figs. 4-12). Nucleolus frequently seen. Pigment granules of small size (< 0.5 μm), roundish, black, numerous (Table I), randomly scattered throughout cytoplasm. Outline of gametocytes irregular (Figs. 4, 6, 12), wavy (Figs. 7, 8), or slightly ameboid (Figs. 9-11), but more frequently the latter. Mature gametocytes are halteridial, they markedly

displace nuclei of erythrocytes laterally (Figs. 9, 10), frequently to envelope of erythrocytes (Fig. 11); such gametocytes predominate in type material. Fully-grown gametocytes markedly displace nuclei of infected erythrocytes, sometimes asymmetrically (Fig. 10), and even to poles of erythrocytes (Fig. 12). Gametocytes in enucleated host cells present in all type preparations, but rare in number (<1% of all gametocytes).

Microgametocytes (*Figs. 13-16):* General configuration as for macrogametocytes with usual haemosporidian sexually dimorphic characters. Pigment granules lighter in color than in macrogametocytes, gathering close to ends of gametocytes. Enucleated host cells present (Fig. 16) with same frequency as for macrogametocytes.

Vector studies

Three closely related lineages (hHIPP26W, hHIPP28W, hHIPP30W, see Fig. 33, box B), which are identical or closely related to lineages of *H. multipigmentatus* recorded in birds, were found in the thoraxes of 3 hippoboscid flies *M. galapagoensis* collected from Galapagos doves on Santiago Island, Santa Fe Island, and Española Island. Because thoraxes of these flies were PCR-positive for parasite DNA, but negative for bird DNA, it is likely that the detected parasite lineages are not from intraerythrocytic gametocytes, but belong to the sporozoite stage of *H. multipigmentatus.* Additionally, 1 thorax was positive for both parasite (lineage hHIPP29W, Fig. 33, box B) and bird DNA, 2 thoraxes were negative for parasite, but positive for bird DNA, and 1 was negative for both parasite and bird DNA. We compared the bird cyt *b* sequences obtained from fly thoraxes to what is available in GenBank™ by using the BLAST algorithm. Our results showed similarities (best

match) of 98 to 100% to a cyt *b* sequence obtained from Galapagos dove (accession number AF251531), showing that insects certainly feed on the doves. These data show that *M. galapagoensis* is a probable natural vector of *H. multipigmentatus*.

Taxonomic summary

Type host: *Zenaida galapagoensis* Gould (Columbiformes, Columbidae).

Type locality: Cueva Norte, Fernandina, Galapagos, Ecuador (0°28.166' S, 91° 50.899' W, approximately 30 m above sea level).

Type specimens: Hapantotype (accession numbers 47725 NS, 47726 NS, intensity of parasitemia is 0.1%, *Zenaida galapagoensis*, Cueva Norte, Fernandina, Galapagos, 00°28.166' S, 91° 50.899' W, lineage hJH003W, collected by G. Valkiūnas, 18 July 2009) is deposited in the Institute of Ecology, Nature Research Centre, Vilnius, Lithuania. Parahapantotypes (accession nos. USNPC 102680, USNPC 102681, G465418, G465419, and 47727 NS, 47728 NS) are deposited in the U. S. National Parasite Collection, Beltsville, Maryland, in the Queensland Museum, Queensland, Australia, and in the Institute of Ecology, Nature Research Centre, Vilnius, Lithuania, respectively.

Additional material: Two blood films (accession numbers USNPC 102682, G465420, intensity of parasitemia is 0.01%, *Zenaida galapagoensis*, Santa Cruz, Charles Darwin Station, 00°44.338' S, 90° 18.108' W, collected by P. G. Parker, 10 July 2009) are deposited in the U. S. National Parasite Collection, Beltsville, Maryland, and in the Queensland Museum, Queensland, Australia, respectively.

DNA sequences: Mitochondrial cyt *b* lineages hJH003W, hJH3B002W, hJH3008W from type material (481, 492, and 481 base pairs, respectively; GenBank™ accession nos. GU296216, GU296215, GU296224, respectively).

Site of infection: Mature erythrocytes; no other data.

Vector: Microlynchia galapagoensis (Diptera, Hippoboscidae) is a probable vector in Galapagos.

Prevalence: In the type locality, the prevalence was 3 of 3 (100%). Overall prevalence in the Galapagos dove in different islands in Galapagos ranges between 36 and 100% (Padilla et al., 2004; Santiago-Alarcon et al., 2008).

Distribution and additional hosts: The lineages hLPMEXW, hCTGUA1W, and hZA16PERUW have been recorded in columbiform birds in Mexico (host is Grey-headed dove *Leptotila plumbeiceps*), Guatemala (ruddy ground-dove *Columbina talpacoti*), and Peru (eared dove *Zenaida auriculata*), respectively. These lineages are closely related to the lineages of *H. multipigmentatus* from the parasite's type material (Fig. 33, box B). *Haemoproteus multipigmentatus* is widely distributed throughout the range of the Galapagos dove in Galapagos and also is transmitted among other species of Columbiformes in countries with warm climates in the New World.

Etymology: The species name reflects presence of numerous pigment granules in mature gametocytes of this parasite.

Remarks

Six species of hemoproteids parasitize birds belonging to Columbiformes (Bennett and Peirce, 1990; Valkiūnas, 2005). *Haemoproteus maccallumi* Novy and

MacNeal, 1904 was also described in columbiform birds. However, the original description of this parasite is based on simultaneous infection of *H. columbae* and *H. sacharovi*, so the name *H. maccallumi* is a partial synonym of both these parasites and thus is invalid (see Novy and MacNeal, 1904; Valkiūnas, 2005). *Haemoproteus multipigmentatus* can be readily distinguished from all these parasites based on the numerous (approximately 40 in average) pigment granules in its mature gametocytes (Table I, Figs. 4-16).

Four species of hemoproteids parasitize doves and pigeons (Figs. 17-32): *H. columbae (*Kruse, 1890), *H. palumbis* (Baker, 1966), *H. sacharovi* (Novy and MacNeal, 1904), and *H. turtur* (Covaleda Ortega and Gállego Berenguer, 1950), so should be distinguished from *H. multipigmentatus.* All these parasites are transmitted by hippoboscid flies and belong to the subgenus *Haemoproteus* (Bennett et al., 1965; Atkinson, 1991; Valkiūnas, 2005). In addition to the number of pigment granules, *H. multipigmentatus* can be readily distinguished from these parasites due to the following features. In gametocytes of *H. columbae,* volutin and pigment granules tend to aggregate into large round compact masses (Figs. 21-22), which frequently exceed 1 μm in diameter in microgametocytes (Figs. 23-24). Mature gametocytes of *H. sacharovi* are highly pleomorphic and possess fine pigment granules (Figs. 29-32), they are outwardly similar to gametocytes of *Leucocytozoon* spp.; average width of fully-grown gametocytes of this parasite is > 5 μm (Valkiūnas, 2005). Mature gametocytes of *H. palumbis* do not displace or only slightly displace nuclei of infected erythrocytes (Figs. 25-28). None of these features is characteristic of *H. multipigmentatus,* which is particularly similar to *H. turtur*, so should be compared

with the latter parasite. Fully-grown gametocytes of *H. turtur* frequently do not touch nuclei of erythrocytes (Figs. 17-20); they frequently possess slightly elongated medium-size (0.5-1 μm) pigment granules and are overfilled with prominent volutin gathered mainly on the ends of the parasites (see Figs. 17-20); these features are not characteristic of *H. multipigmentatus*. Additionally, based on material from type vertebrate hosts, area of macrogametocyte nuclei in *H. multipigmentatus* is approximately half the size of those in *H. turtur* ($P < 0.001$).

Phylogenetic relationships of parasites

All positively identified species of avian hemoproteids are clearly distinguishable in the phylogenetic tree (Fig. 33), which corresponds with their morphological differences. Because parasites of the lineages recorded in the type material of *H. multipigmentatus*, and all other lineages of hemoproteids in the Galapagos dove are closely related (Fig. 33, box B) and are indistinguishable based on morphology of their blood stages, we consider all these lineages as intraspecies genetic variation of the same morphospecies, i. e., *H. multipigmentatus*.

Genetic distance in cyt *b* gene among different lineages of *H. multipigmentatus* ranges between 0.2% and 3.9%; and it is < 2.5% for the great majority of lineages of this parasite (Fig. 33, box B). Genetic distance between all recorded lineages of *H. multipigmentatus* and the lineages of hippoboscid transmitted *H. (Haemoproteus) columbae* ranges between 7.5% and 10.6%. Genetic differences among lineages of *H. multipigmentatus* and the lineages of positively identified species of ceratopogonid transmitted *Haemoproteus (Parahaemoproteus)* spp. (Fig. 33, box A) ranges between 8.6% and 15.7%.

DISCUSSION

Haemoproteus multipigmentatus is attributed to the subgenus *Haemoproteus* because of 2 sets of our data. First, cyt *b* lineages of this parasite cluster well with the lineages of *H. (Haemoproteus) columbae* (Fig. 33, box B), but not to the lineages of other avian species of the subgenus *Parahaemoproteus* (Fig. 33, box A). Hemoproteids of the subgenera *Haemoproteus* and *Parahaemoproteus* are transmitted by different groups of dipteran vectors (species of Hippoboscidae and Ceratopogonidae, respectively); and they undergo markedly different sporogony in the vectors (see Bennett et al., 1965; Garnham, 1966; Atkinson, 1991; Valkiūnas, 2005), so usually appear in different well-supported clades in phylogenetic trees (Martinsen et al., 2008; Santiago-Alarcon et al., 2009; Iezhova et al., 2010). Second, the same and closely related lineages of *H. multipigmentatus* were also detected in thoraxes of hippoboscid flies *M. galapagoensis*, which were collected from Galapagos doves. Because thoraxes of 3 flies were PCR-positive for parasite DNA but negative for avian DNA, these lineages likely belong to sporozoite stage of *H. multipigmentatus*. In avian hemosporidians, sporozoites represent the only sporogonic stage, which present in thoraxes of dipteran vectors, mainly in salivary glands (Garnham, 1966; Atkinson, 1991; Valkiūnas, 2005). It is important to note that biting midges, vectors of *Haemoproteus (Parahaemoproteus)* species were not collected in mosquito traps at the type locality of *H. multipigmentatus* (G. Valkiūnas, unpubl. obs.); this is a very dry desert site. The traps were covered with a fine mesh and were satisfactory for catching of biting midges. It is unlikely that biting midges, which require relatively high humidity for active life (Glukhova, 1989), are the vectors of

this hemoproteid at this study site. It is most probable that *H. multipigmentatus* is transmitted by the hippoboscid fly *M. galapagoensis*, which is prevalent on the Galapagos dove and parasitizes this bird throughout the archipelago, including dry sites without permanent freshwater, as in Wolf Island (D. Santiago-Alarcon, unpubl. obs.). Thus, these results support the role of *M. galapagoensis* as the natural vector of *H. multipigmentatus* in the Galapagos archipelago. Detection of oocysts in mid-gut and sporozoites in salivary glands of the flies, ideally followed by experimental infection of uninfected doves by sporozoites, are needed to provide unequivocal support that *M. galapagoensis* is the vector.

It should be noted that it is still unclear if the phylogenetic analysis of cyt *b* genes can be applied for molecular identification of subgeneric position of all hemoproteid species. This is mainly because the phylogenetic position of the majority of hippoboscid-transmitted morphospecies of subgenus *Haemoproteus* remains unknown. Surprisingly, *H. (Haemoproteus) turtur*, a common parasite of doves in the Old World, appeared in the *Parahaemoproteus* clade in different phylogenies of avian hemosporidians (Martinsen et al., 2008; Santiago-Alarcon et al., 2009; see Fig. 33, box A). Because this parasite completes sporogony in hippoboscid flies and belongs to the subgenus *Haemoproteus* (Rashdan, 1998; Valkiūnas, 2005), it might be that molecular identification of hippoboscid-transmitted hemoproteids using currently applied molecular markers cannot be applied to all species of these parasites. Sequences of other positively identified hemoproteids that are transmitted by hippoboscids, as well as additional sequences of *H. turtur*, are needed to clarify this issue. Further work to increase the number of precise linkages between

hemosporidian DNA lineages with their morphospecies, particularly of hippoboscid-transmitted parasites of the subgenus *Haemoproteus*, is an important task. This study adds *H. multipigmentatus* to the phylogenetic studies of the hippoboscid transmitted hemoproteids.

We used mainly positively identified morphospecies of avian hemoproteids in the phylogenetic analysis (Fig. 33). Genetic distances between all cyt *b* lineages of *H. multipigmentatus* and the lineages of *H. columbae* is > 7.5%. Genetic divergence among lineages of all positively identified morphospecies of hemosporidian parasites is > 4%; it is > 5% for the great majority of the readily distinguishable morphospecies (see Fig. 33), implying that genetic divergence of > 5% can be used for the better understanding of phylogenetic trees based on the fragment of the cyt *b* gene used in the present study. This conclusion supports hypothesis of Hellgren et al. (2007) that haemosporidian species with a genetic distance greater than 5% in the mitochondrial cyt *b* gene are expected to be morphologically differentiated. This has been shown to be true for many readily distinguishable morphospecies of avian hemosporidian parasites of the genera *Haemoproteus*, *Plasmodium*, and *Leucocytozoon* (but see also Valkiūnas et al., 2009; Iezhova et al., 2010). Accumulation of information on this subject is useful because it provides additional data for the better understanding of phylogenetic trees based on a certain fragment of the cyt *b* gene.

It is interesting to note that the lineage hCB4ECU, which was obtained from the blood of an Ecuadorian ground dove *Columbina buckleyi* in mainland Ecuador, clusters with lineages of hemoproteids of the subgenus *Haemoproteus* (Fig. 33, box B). Because genetic distance among the lineage hCB4ECU and other lineages of *H.*

multipigmentatus and *H. columbae* is > 7%, it is possible that the former lineage belong to different morphospecies. However, when parasite PCR products from the same sample were sequenced using the primers developed by Perkins and Schall (2002), which amplify the other section of mitochondrial cyt *b* gene of hemoproteids, the lineage hCB4ECU is equal to a parasite lineage GDE9 obtained from the endemic Galapagos doves and it is similar to several other parasite lineages retrieved from endemic Galapagos doves as well, e.g., lineages, GDE23, GDMA20, and GDSF9 (see Santiago-Alarcon et al., 2009).

We think this situation can be explained due to possibly an undetected mixed infection of hCB4ECU and *H. multipigmentatus* and primer bias when amplifying different sections of the cyt *b* gene of these parasites. PCR frequently does not read mixed hemosporidian infections (Valkiūnas et al., 2006), which are common in mainland birds, and different primers might amplify different parasites if they have a better match during a mixed infection with 2 or more related organisms (Cosgrove et al., 2006; Szöllősi et al., 2008). This issue could be settled if morphological material was available. Unfortunately, we do not have access to such information, which strongly points to the need of using both morphology and gene markers in studies of hemosporidian parasites, particularly in wildlife. Importantly, blood films, which are used for microscopic examination, should be prepared, stained, and examined properly (see Valkiūnas, Iezhova, Križanauskienė et al., 2008); that is not a case in some recent evolutionary biology studies. In addition, it is important that avian hemosporidian researchers standardize the sequence length and genetic marker of choice for hemosporidian parasite identification. Until now, Waldenström et al.'s

(2004) primers have been used successfully for this task. Moreover, it seems that when it comes to the mitochondrial cyt *b* gene, it does not matter if longer or shorter fragments are used (Hellgren et al., 2007). However, the problem of the lineage hCB4ECU raised here suggests that more discussion among researchers is needed to clearly establish the sequence length and number of genes used for identification of hemosporidian parasites at different taxonomic levels.

All recorded lineages of *H. multipigmentatus* (Fig. 33, box B) are widespread in Galapagos; they show no differences in genetic structure across the archipelago (Santiago-Alarcon et al., 2009). Using primers described by Perkins and Schall (2002), Santiago-Alarcon et al. (2009) found several hemoproteid lineages, which are closely related to lineages of *H. multipigmentatus*. These lineages were found in 10 species of birds in the New World, i.e., the Zenaida dove (*Zenaida aurita*; Caribbean Islands), eared dove (Ecuador and Venezuela), Pacific dove (*Z. meloda*; Peru), Ecuadorian ground dove (*Columbina buckleyi*; Ecuador), croaking ground dove (*C. cruziana*; Ecuador), ruddy ground dove (*C. talpacoti*; Guatemala), rock dove (Ecuador), grey-headed dove (*Leptotila plumbeiceps*; Mexico), Inca dove (*Scardafella inca*; Guatemala), and ruddy quail-dove (*Geotrygon montana*; Ecuador). Further investigation of blood stages of the parasites is needed to prove if any of them belong to *H. multipigmentatus.*

The Galapagos dove is endemic to Galapagos and is widespread in the archipelago (Santiago-Alarcon et al., 2006; Santiago-Alarcon and Parker, 2007), and so serves as a convenient model organism in studies of ecology and evolution of parasitic diseases in geographically restricted, but highly mobile, hosts (Parker et al.,

2006; Santiago-Alarcon et al., 2009). The present study shows that *H. multipigmentatus* is a highly prevalent and widespread hemoproteid of the Galapagos dove. Because the same, or closely related, lineages of *H. multipigmentatus* are present in several species of columbiform birds in the New World, this parasite certainly has a wide range of transmission, as is the case with some other species of avian hemoproteids (Bishop and Bennett, 1992; Valkiūnas, 2005; Bensch et al., 2009). To date, *H. multipigmentatus* and its lineages have been recorded in countries with warm climates in the New World. Recent genetic studies suggest that *H. multipigmentatus* is a relatively new arrival to the archipelago probably from different continental dove populations (Santiago-Alarcon et al., 2009). Closely related lineages of *H. multipigmentatus* have been recorded in continental populations of the eared dove; this bird is widely distributed in South America and also has been recorded as a vagrant species in Galapagos (Curry and Stoleson, 1988). It is possible that vagrant eared doves could have naturally introduced *H. multipigmentatus* into the Galapagos Islands. Rock doves were also repeatedly introduced to the archipelago, so might also be a source of infection for the Galapagos dove. However, lineages of *H. multipigmentatus* have not been recorded in the rock doves in Galapagos (P. Parker, unpubl. obs.) and have not been documented in continental populations of this bird. Thus, the rock dove, which was completely eradicated from the archipelago in 2002, seems less probable source of infection for the Galapagos dove. Additional studies of hemoproteids in continental populations of columbiform birds are needed to understand the origin of *H. multipigmentatus* in Galapagos.

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Table I. Morphometry of host cells and mature gametocytes of *Haemoproteus multipigmentatus* sp. nov. from the Galapagos dove *Zenaida galapagoensis*.

Feature	Measurements (μm) *
Uninfected erythrocyte	
Length	10.5-12.2 (11.3±0.5)
Width	6.4-7.9 (7.1±0.3)
Area	58.2-72.0 (64.1±4.0)
Uninfected erythrocyte nucleus	
Length	4.1-6.1 (5.2±0.5)
Width	2.1-2.9 (2.5±0.2)
Area	9.5-13.2 (11.0±1.1)
Macrogametocyte	
Infected erythrocyte	
Length	11.5-14.5 (13.1±0.9)
Width	4.9-7.3 (6.5±0.5)
Area	59.6-76.7 (69.6±5.3)
Infected erythrocyte nucleus	
Length	4.4-5.9 (5.2±0.4)
Width	2.3-3.2 (2.8±0.3)
Area	10.1-14.6 (12.0±1.2)

Gametocyte	
Length	13.7-20.4 (16.4±1.6)
Width	2.6-4.1 (3.3±0.4)
Area	37.7-54.8 (47.8±5.3)
Gametocyte nucleus	
Length	2.0-2.9 (2.4±0.2)
Width	1.2-2.2 (1.7±0.3)
Area	2.5-4.4 (3.4±0.5)
Pigment granules	33.0-54.0 (43.4±5.2)
NDR†	0.0-0.7 (0.2±0.2)
Microgametocyte	
Infected erythrocyte	
Length	11.1-14.1(13.1±0.8)
Width	5.8-7.6 (6.7±0.5)
Area	54.3-80.8 (70.3±7.0)
Infected erythrocyte nucleus	
Length	4.7-5.7 (5.2±0.2)
Width	2.2-3.3 (2.6±0.3)
Area	8.7-13.1 (11.0±1.0)
Gametocyte	
Length	12.4-16.2 (14.6±0.9)

Width	2.7-3.9 (3.3±0.4)
Area	42.1-55.3 (48.4±4.7)
Gametocyte nucleus	
Length	5.1-9.2 (6.3±1.0)
Width	1.7-3.1 (2.6±0.4)
Area	9.9-18.3 (14.9±2.4)
Pigment granules	30.0-48.0 (38.9±5.0)
NDR	0.0-0.6 (0.4±0.2)

* All measurements (n=21) are given in micrometers. Minimum and maximum values are provided, followed in parentheses by the arithmetic mean and standard deviation.

† NDR = nucleus displacement ration according to Bennett and Campbell (1972).

FIGURE LEGENDS

FIGURES 1-16. *Haemoproteus (Haemoproteus) multipigmentatus* sp. nov. from the blood of the Galapagos dove *Zenaida galapagoensis*. (**1, 2**) Young gametocytes. (**3-12**) Macrogametocytes. (**13-16**) Microgametocytes. Long arrows – nuclei of parasites. Short arrows - unfilled spaces among gametocytes and envelope and nuclei of infected erythrocytes. Arrow heads – azurophilic granules. (1, 2, 4-16) Giemsa-stained thin blood films. (3) Field-stained thin blood films. Bar = 10 μm.

FIGURES 17-32. Mature gametocytes of widespread hippoboscid-transmitted species of hemoproteids. (**17-20**) *Haemoproteus (Haemoproteus) turtur* from the blood of *Streptopelia turtur;* (**21-24**) *Haemoproteus (Haemoproteus) columbae* from the blood of *Columba livia*; (**25-28**) *Haemoproteus (Haemoproteus) palumbis* from the blood of *Columba palumbus;* (**29-32**) *Haemoproteus (Haemoproteus) sacharovi* from the blood of *Zenaida macroura.* (17, 18, 21, 22, 25, 26, 29, 30**)** Macrogametocytes. (19, 20, 23, 24, 27, 28, 31, 32) Microgametocytes. Long arrows – nuclei of parasites. Short arrows – unfilled spaces among gametocytes and nuclei of infected erythrocytes. Arrow heads – volutin granules. Giemsa-stained thin blood films. Bar = 10 μm.

FIGURE 33. Bayesian majority-rule consensus phylogeny of 48 mitochondrial cytochrome *b* lineages of avian hemosporidians and 2 lineages of *Leucocytozoon shoutedeni* used as an outgroup. GenBank™ accession numbers of sequences and names of lineages are given before parasite species names. Gray boxes indicate group

of closely related lineages of hemoproteids belonging to the subgenera *Parahaemoproteus* (**A**) and *Haemoproteus* (**B**). Lineages in bold face represent parasite lineages recovered from the hippoboscid fly *Microlynchia galapagoensis*, the probable vector of *Haemoproteus* (*H.*) *multipigmentatus* in the Galapagos archipelago. Values on branches represent the Bayesian posterior probabilities for the different nodes; scale bar is given in percentage.

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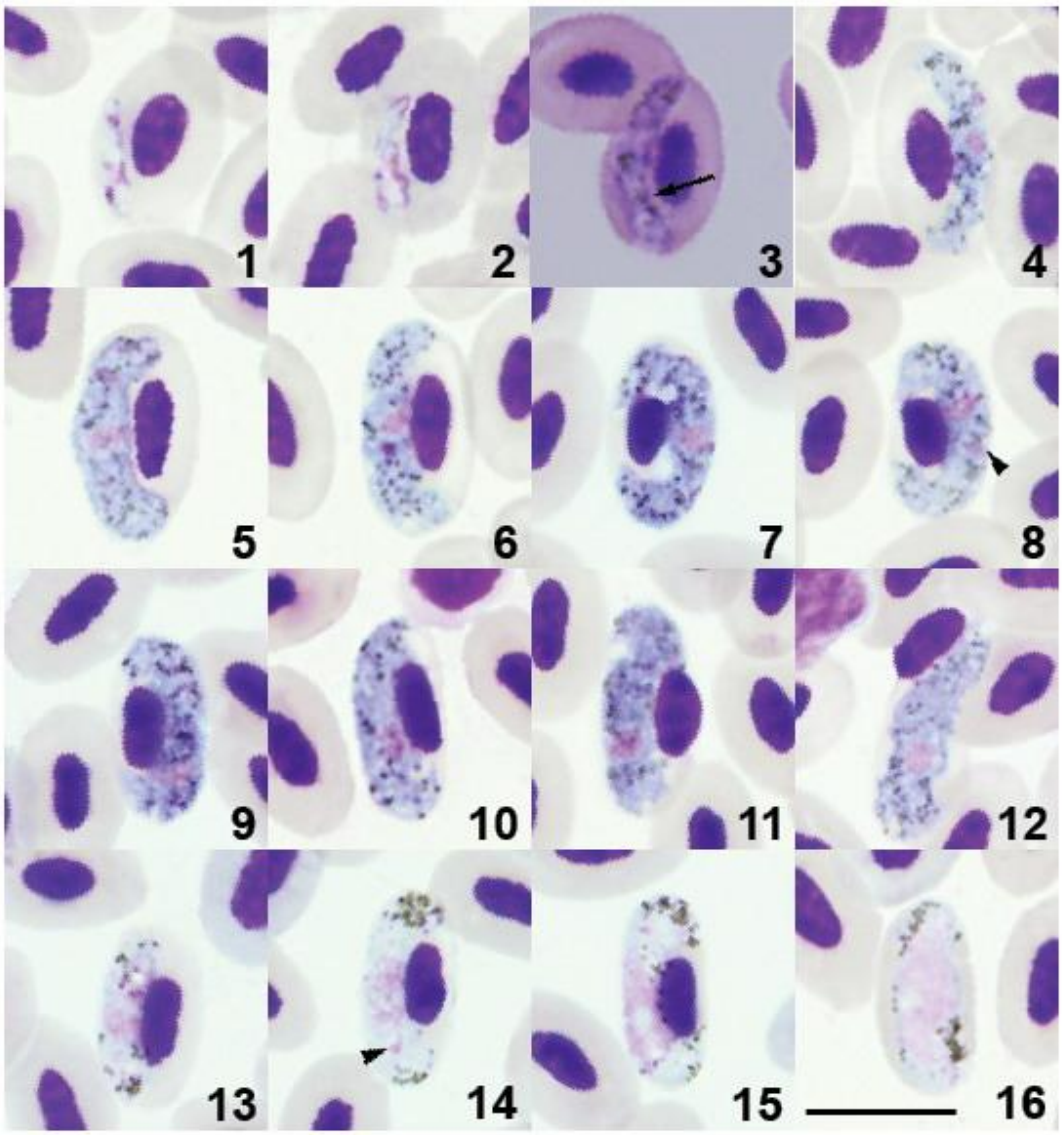
Figure 1-16:



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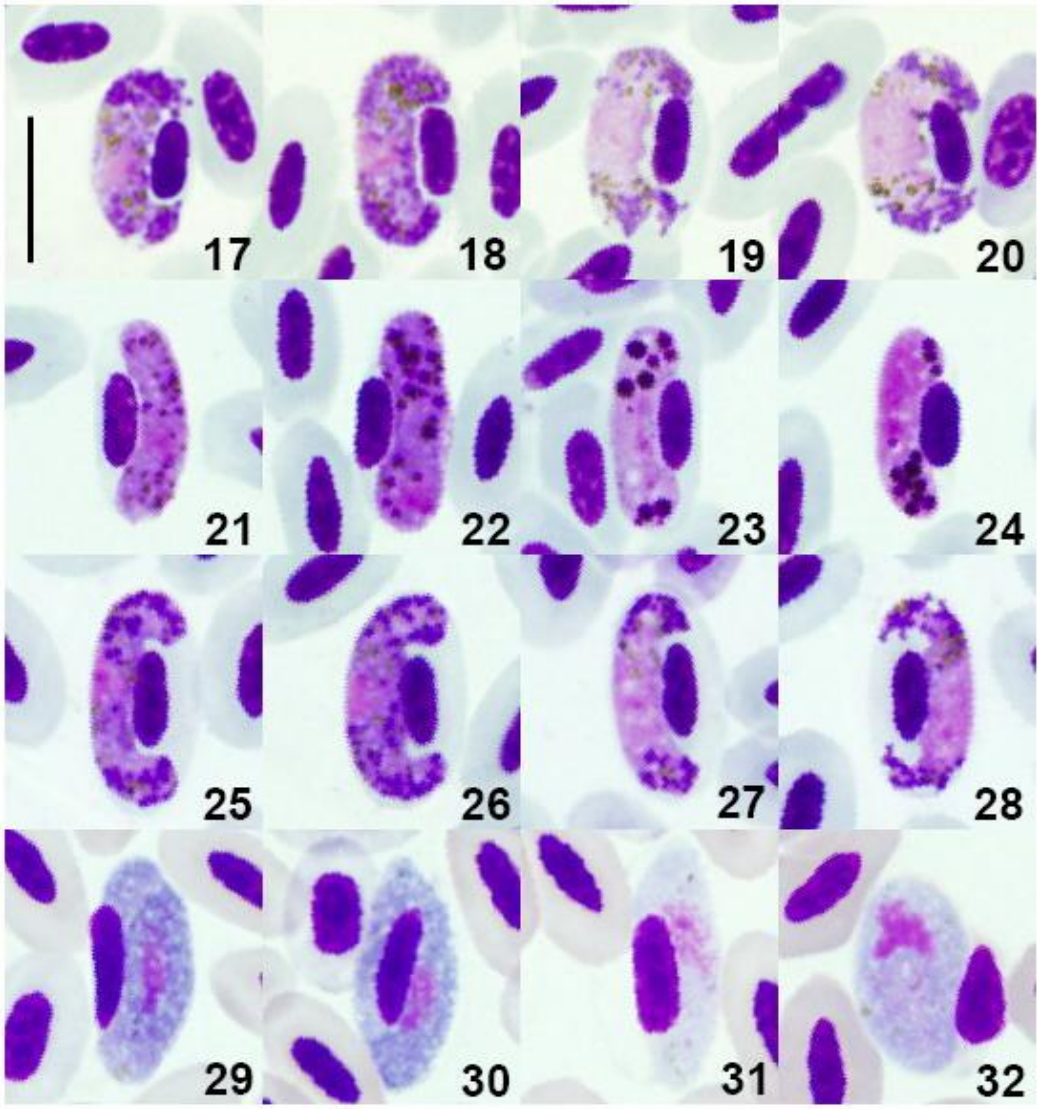
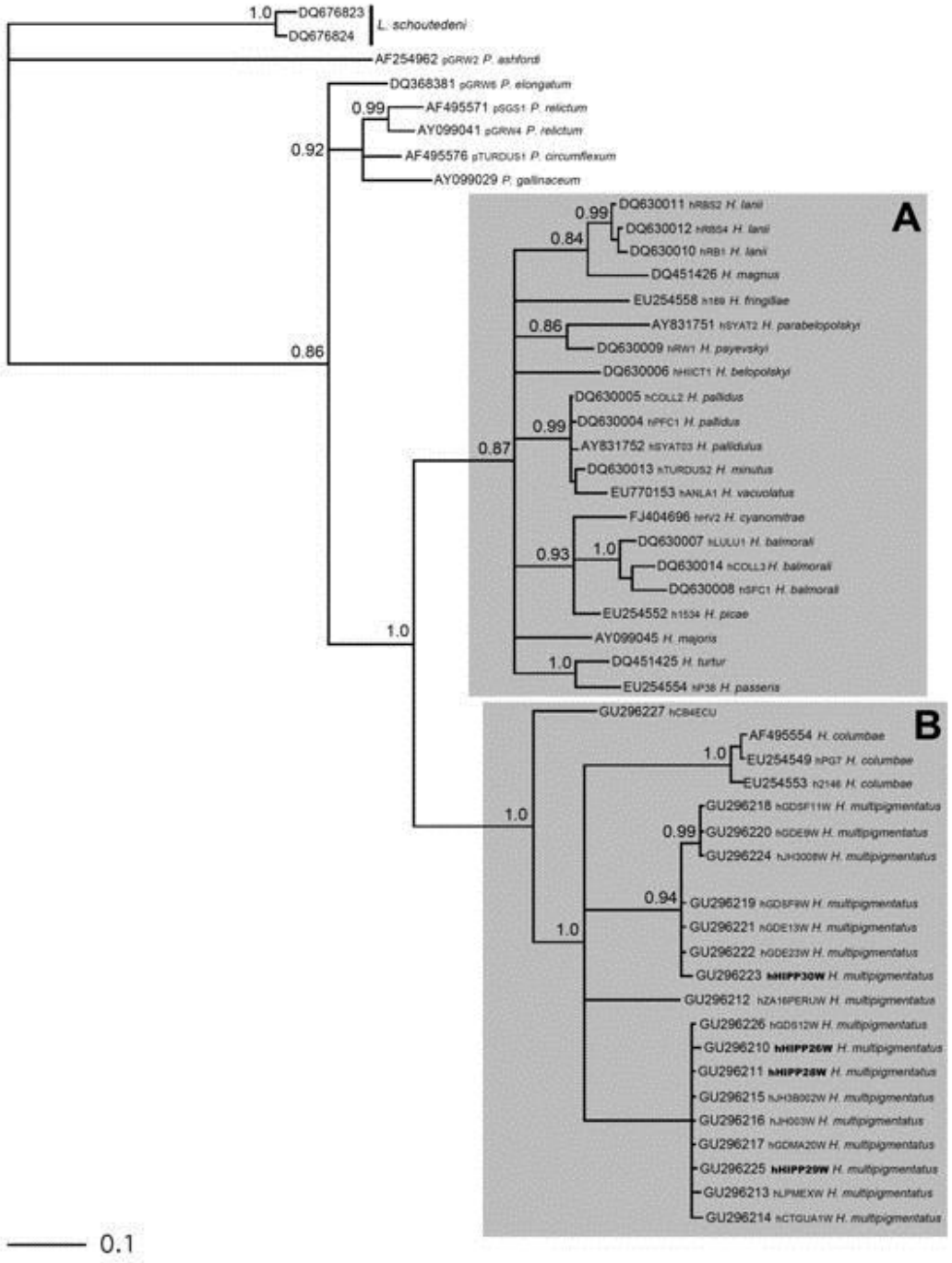


Figure 33:


1.0
DQ676823
DQ676824
L. schoutedeni
AF254962 pGRW2 P. ashfordi
DQ368381 pGRW6 P. elongatum
0.99
AF495571 pGGS1 P. relictum
AY099041 pGRW4 P. relictum
0.92
AF495576 pTURDUS1 P. circumflexum
AY099029 P. gallinaceum
A
0.99
DQ630011 hRBS2 H. lanii
DQ630012 hRBS4 H. lanii
0.84
DQ630010 hRB1 H. lanii
DQ451426 H. magnus
EU254558 h169 H. fringillae
0.86
AY831751 hSYAT2 H. parabelopolskyi
DQ630009 hRW1 H. payevskyi
0.86
DQ630006 hHIICT1 H. belopolskyi
DQ630005 hCOLL2 H. pallidus
0.99
DQ630004 hPFC1 H. pallidus
0.87
AY831752 hSYAT03 H. pallidulus
DQ630013 hTURDUS2 H. minutus
EU770153 hANLA1 H. vacuolatus
FJ404696 hHV2 H. cyanomitrae
DQ630007 hLULU1 H. balmorali
0.93
1.0
DQ630014 hCOLL3 H. balmorali
DQ630008 hSFC1 H. balmorali
EU254552 h1534 H. picae
1.0
AY099045 H. majoris
DQ451425 H. turtur
1.0
EU254554 hP38 H. passeris
B
GU296227 hCB4ECU
AF495554 H. columbae
1.0
EU254549 hPGT H. columbae
EU254553 h2146 H. columbae
1.0
GU296218 hGDSF11W H. multipigmentatus
0.99
GU296220 hGDE9W H. multipigmentatus
GU296224 hJH3008W H. multipigmentatus
0.94
GU296219 hGDSF9W H. multipigmentatus
1.0
GU296221 hGDE13W H. multipigmentatus
GU296222 hGDE23W H. multipigmentatus
GU296223 hHIPP30W H. multipigmentatus
GU296212 hZA16PERUW H. multipigmentatus
GU296226 hGD512W H. multipigmentatus
GU296210 hHIPP26W H. multipigmentatus
GU296211 hHIPP28W H. multipigmentatus
GU296215 hJH3B002W H. multipigmentatus
GU296216 hJH003W H. multipigmentatus
GU296217 hGDMA20W H. multipigmentatus
GU296225 hHIPP29W H. multipigmentatus
GU296213 hLPMEXW H. multipigmentatus
GU296214 hCTGUA1W H. multipigmentatus
0.1

II. **ABSTRACT:** *Haemoproteus (Haemoproteus) jenniae* n. sp. (Haemosporida, Haemoproteidae) is described from the Galapagos bird, the swallow-tailed gull *Creagrus furcatus* (Charadriiformes, Laridae), based on the morphology of its blood stages and segments of the mitochondrial cytochrome *b* (cyt *b*) gene. The most distinctive features of *H. jenniae* development are the circumnuclear gametocytes occupying all cytoplasmic space in infected erythrocytes and the presence of advanced growing gametocytes, in which the pellicle is closely appressed to the erythrocyte envelope, but does not extend to the erythrocyte nucleus. This parasite is distinguishable from *H. larae*, which produces similar gametocytes and parasitizes closely related species of Laridae. *Haemoproteus jenniae* can be distinguished from *H. larae*, primarily due to 1) the predominantly amoeboid outline of young gametocytes, 2) diffuse macrogametocyte nuclei, which do not possess distinguishable nucleoli, 3) consistent size and shape of pigment granules, and 4) absence of rod-like pigment granules from gametocytes. Additionally, fully-grown gametocytes of *H. jenniae* cause both the marked hypertrophy of infected erythrocytes in width and the rounding up of the host cells, which is not a case in *H. larae*. Phylogenetic analyses identify the DNA lineages that are associated with *H. jenniae*, and show that this parasite is more closely related to the hippoboscid-transmitted (Hippoboscidae) species than to the *Culicoides* spp.-transmitted (Ceratopogonidae) species of avian hemoproteids. Genetic divergence between morphologically well-differentiated *H. jenniae* and the hippoboscid-transmitted *Haemoproteus iwa*, the closely related parasite of frigatebirds (Fregatidae, Pelecaniformes), is only 0.6%; cyt *b* sequences of these parasites differ only by 1 base

pair. This is the first example of such a small genetic difference between species of the subgenus *Haemoproteus*. This corroborates the conclusion that hippoboscid-transmitted *Haemoproteus* parasites infect not only columbiform birds, but also infect marine birds belonging to Pelecaniformes and Charadriiformes. We conclude that the vertebrate host range should be carefully used in identification of subgenera of avian *Haemoproteus*, and the phylogenies based on cyt *b* gene provide evidence for determining the subgeneric position of avian hemoproteids.

INTRODUCTION

Species of *Haemoproteus* (Haemosporida, Haemoproteidae) are cosmopolitan dipteran-borne hemosporidian parasites, some of which are responsible for severe pathology in birds (Miltgen et al., 1981; Atkinson et al., 1986; Cardona et al., 2002). These parasites affect host fitness (Nordling et al., 1998; Marzal et al., 2005; Valkiūnas, 2005; Møller and Nielsen, 2007) and even might cause lethal disease in non-adapted birds. The mortality associated with hemoproteid infection has been documented in zoos and private aviaries in America (Ferrell et al., 2007) and Europe (Olias et al., 2011) and is related to the insufficiently investigated pathology caused by tissue stages of the parasites, when death of the host occurs before the production of blood stages. Such infections are difficult to diagnose both by microscopy and PCR-based methods (Valkiūnas, 2011). Avian hemoproteids warrant more research, not only in parasitology and evolutionary biology, but also in conservation projects.

Until recently, parasites of the subgenus *Haemoproteus* (*Haemoproteus*) were understood to only infect doves (Columbiformes); however, seabirds, particularly frigatebirds (*Fregata* spp.), were found infected with a morphologically and

genetically similar species (Levin et al. 2011). *Haemoproteus iwa*, the species infecting frigatebirds, is vectored by hippoboscid flies, as are the *Haemoproteus* (*Haemoproteus*) species that infect doves. This discovery of the greater host breadth of *H.* (*Haemoproteus*) spp., which shares a common vector group, namely species of the Hippoboscidae, is consistent with the overall pattern of vector group driving the topology of the phylogenetic tree for hemosporidians (Martinsen et al., 2008). Avian hippoboscid flies are obligate parasites of birds, spending much of their time on an individual host or host species. Therefore, there is opportunity for specialization and diversification. With this in mind, it is likely that there is a diversity of *H.* (*Haemoproteus*) parasites vectored by hippobscid flies that have not been collected and described.

As part of an ongoing study of the evolutionary biology of pathogens in the Galapagos Islands, blood samples from a Galapagos gull, the swallow-tailed gull *Creagrus furcatus* (Charadriiformes, Laridae), were collected. One novel species of *Haemoproteus* (Haemosporida, Haemoproteidae) was found during this study. This parasite is described here using data on the morphology of its blood stages and partial sequences of the mitochondrial cytochrome *b* (cyt *b*) gene. We identify the DNA lineages that are associated with this parasite and show that it is more closely related to hippoboscid-transmitted species than to the *Culicoides* (Ceratopogonidae) spp.-transmitted species of avian hemoproteids. We also discuss opportunities to use phylogenies based on cyt *b* gene sequences in identification of subgeneric position of avian hemoproteids and provide new information on the possible host range of the hippoboscid-transmitted species of avian *Haemoproteus*.

MATERIAL AND METHODS

Collection of blood samples

Blood samples from swallow-tailed gulls were collected during dry season on the islands Genovesa (July 2003) and Española (June 2010) in Galapagos, Ecuador. Only 1 bird was samples on Genovesa. Of the 30 birds sampled on Española, 29 were adults, nearly half of which (13/30) were breeding and only one juvenile bird was sampled. While sampling these birds, one individual hippoboscid fly of unidentified species was seen, but we were unable to collect it. Birds were measured and one or two drops of blood were collected by puncturing the brachial or medial metatarsal vein and placed in 500 µl of lysis buffer for subsequent molecular analysis. The samples were held at ambient temperature in the field and later at 4 C in the laboratory.

Three or four blood films were prepared from each bird. Blood films were air-dried within 5-10 sec after their preparation. In humid environments, we used a battery-operated fan to aid in the drying of the blood films. Slides were fixed in methanol in the field and then stained with Giemsa in the laboratory. Blood films were examined for 10-15 min at low magnification (×400) and then at least 100 fields were studied at high magnification (×1,000). Intensity of infection was estimated as a percentage by counting of the number of parasites per 1,000 red blood cells or per 10,000 red blood cells if infections were light, i.e., < 0.1%, as described by Godfrey et al. (1987). To determine possible presence of simultaneous infections with other hemosporidian parasites in the type material of new species, the entire blood films

from hapantotype and parahapantotype series were examined microscopically at low magnification.

Morphological analysis

An Olympus BX61 light microscope (Olympus, Tokyo, Japan) equipped with Olympus DP70 digital camera and imaging software AnalySIS FIVE (Olympus Soft Imaging Solution GmbH, Münster, Germany) was used to examine slides, to prepare illustrations, and to take measurements. The morphometric features studied (Table I) are those defined by Valkiūnas (2005). Morphology of *Haemoproteus jenniae* was compared with the voucher specimens of *Haemoproteus larae* from its type host, the black-headed gull *Chroicocephalus ridibundus*, sampled from the type locality in Southeast Kazakhstan (blood film accession no. 1525.Az 86 in the Collection of Institute of Ecology, Nature Research Centre, Vilnius, Lithuania). Student's *t*-test for independent samples was used to determine statistical significance between mean linear parameters. A *P*-value of 0.05, or less, was considered significant.

DNA extraction, PCR amplification, and sequencing

Phenol-chloroform extraction techniques were used to isolate DNA from blood (Sambrook et al., 1989). Parasite DNA was amplified by polymerase chain reaction (PCR) targeting a region of the parasite mitochondrial cyt *b* gene. In each reaction, both a positive control (frigatebird, infected with *H. iwa*) and a negative control were used and all samples that amplified parasite DNA were tested again for confirmation. The PCR primers used were HAEMNF and HAEMNR2, followed by a re-amplification reaction using HAEMF and HAEMR2 (Waldenström et al., 2004). Reactions were performed using Takara Ex taq polymerase and accompanying

reagents (Takara Bio Inc., Japan) and reaction conditions can be found in Levin et al. (2011). The initial reaction (HAEMNF and HAEMR2) included one microliter of undiluted DNA, and half a microliter of the resulting amplicon was used as the template for the internal reaction. PCR products were purified using Exonuclease I (#M0289S, New England Bio Labs Inc., Ipswich, MA) and Antarctic Phosphotase (#M0293S, New England Bio Labs Inc.) Approximately 480 base pairs (bp) of double-stranded DNA was sequenced at the University of Missouri – St. Louis using an Applied Biosystems 3100 DNA Analyzer with BigDye Terminator v3.1 Cycle Sequencing chemistry.

Phylogenetic analysis

DNA sequences were assembled and edited in Seqman 4.0 (DNASTAR, USA), aligned by eye, and added to a dataset containing cyt *b* sequence data of previously identified hemosporidian parasites obtained from GenBank (accession numbers can be found on the phylogenetic tree, Figure 29). The best-fit model of evolution, GTR + G, was determined using jMODELTEST (version 0.1.1) (Guindon and Gascuel, 2003; Posada, 2008). Treefinder (Jobb et al., 2004) was used to reconstruct a maximum likelihood phylogeny and bootstrap analysis. The sequence divergence among lineages was calculated in MEGA (version 5.05) using a Jukes-Cantor model of substitution in which all substitutions were weighted equally.

RESULTS

With the exception of one DNA sequence from a gull sampled in 2003, the results refer to samples collected in 2010. Only *Haemoproteus* parasites were found in the investigated birds both by microscopic examination and PCR-based

diagnostics. Overall prevalence of infection was 8 of 30 (26.7%). One infection was from a bird that had no obvious mate or nest at the time of capture, and one infection was found in a juvenile bird. Other reported infections were from adults at some stage of breeding (paired with nest, egg, chick). Breeding is not necessarily synchronous in this species or at the study sites; it is difficult to determine whether birds without nests, eggs, or chicks will breed or are roosting at the site.

DESCRIPTION

***Haemoproteus (Haemoproteus) jenniae* n. sp.**

(Figs. 1-16, Table I)

Young gametocytes (Figs. 1-4): Develop in mature erythrocytes. Earliest forms seen anywhere in infected erythrocytes, but more frequently recorded in a position sub-polar (Figs. 1, 4) or lateral (Fig. 2) to erythrocyte nuclei. Advanced gametocytes extend longitudinally along nuclei of erythrocytes, but do not adhere to nuclei (Figs. 3, 4). Growing gametocytes, which exceed length of erythrocyte nuclei, usually do not touch both envelope and nuclei of erythrocytes along entire margin (Figs. 3, 4), a characteristic feature in the development of this species. Nuclear material is diffuse and gathered along periphery in the earliest gametocytes (Figs. 1, 2); it remains diffuse with unclear boundaries in advanced forms (Figs. 3, 4). A clearly visible unstained space resembling a vacuole is present in central part of early gametocytes (Figs. 1, 2); this space decreases in size in advanced gametocytes (Fig. 3). One large vacuole is present in many advanced gametocytes (Fig. 4). Pigment granules are small (< 0.5 μm), and can be grouped in a focus (Fig. 4). Outline of

growing gametocytes is wavy (Fig. 1), irregular (Figs. 3, 4), or ameboid (Fig. 2). The influence of gametocytes on infected erythrocytes is not pronounced (Figs. 1-4).

Macrogametocytes (Figs. 5-12): Develop in mature erythrocytes. Cytoplasm blue, homogenous in appearance, contains small vacuoles, which tend to merge together in advanced gametocytes and to form large (up to 3 μm in diameter) vacuole-like spaces, which are usually located close to one end of gametocytes (Fig. 8). Volutin granules not seen. Gametocytes grow around nuclei of erythrocytes, do not displace nuclei laterally; are closely associated with envelope of erythrocytes, but not with their nuclei (Figs. 5-11). Growing gametocytes either touch the nuclei of erythrocytes only in several points or do not touch them at all, and, as a result, unfilled spaces of irregular shape ('clefts') are present between gametocytes and nuclei. Such 'clefts' disappear in fully-grown gametocytes, which completely encircle erythrocyte nuclei and are closely appressed both to nuclei and envelope of erythrocytes occupying all cytoplasmic space in the erythrocytes (Fig. 12). Circumnuclear forms (Figs. 11, 12) common. Parasite nucleus diffuse, of central or sub-central position, markedly irregular in shape with unclear boundaries (Figs. 5-11), thus difficult to measure, which is a rare character of hemoproteids. Nucleolus not observed. Pigment granules predominantly roundish, occasionally slightly oval in shape, of medium size (0.5-1 μm), mostly randomly scattered throughout cytoplasm (Figs. 5, 10-12), but sometimes grouped (Fig. 9). In the majority of gametocytes, pigment granules are of consistent size and shape, a characteristic feature in this species (Figs. 5-12). Outline of growing gametocytes amoeboid, with prominent indentations on the gametocyte side located towards the erythrocyte nuclei (Figs. 5, 7-

10); it is entire in fully-grown gametocytes (Fig. 12). Nucleus of infected erythrocytes not displaced or only slightly displaced laterally (Table I), but erythrocytes are rounded up, and are significantly hypertrophied in width and area ($P < 0.001$ for both these features in comparison to uninfected erythrocytes). Advanced gametocytes slightly rotate the nuclei of infected erythrocytes (between 5-15%) to the normal axis (Figs. 5, 10, 12).

Microgametocytes (*Figs. 13-16):* General configuration and main features as for macrogametocytes with usual hemosporidian sexually dimorphic characters.

Taxonomic summary

Type host: Swallow-tailed gull *Creagrus furcatus* (Neboux, 1848) (Charadriiformes, Laridae).

Type locality: The type material was collected from a nesting swallow-tailed gull in a mixed-species seabird colony at Punta Cevallos on the island of Española (1°20´S, 89°40´W, close to sea level), Galapagos, Ecuador.

Type specimens: Hapantotype (accession number 47781 NS, intensity of parasitemia is approximately 0.003%, lineage STGGAL1, GenBank accession no. JN827318, *C. furcatus,* Punta Cevallos, Española, 1°20´S, 89°40´W, collected by I. Levin, 28 June 2010) was deposited in the Institute of Ecology, Nature Research Centre, Vilnius, Lithuania. Parahapantotypes (accession no. USNPC 104882.00 and G465491, other data as for the hapantotype) were deposited in the U. S. National Parasite Collection, Beltsville, USA and in the Queensland Museum, Queensland, Australia, respectively.

Additional material: The samples of whole blood from the type host (original field numbers are STG26-STG55) and additional blood film preparations (slide numbers STG26-STG55, other data as for the type material) were deposited in Patricia Parker's molecular ecology laboratory at the University of Missouri – St. Louis, St. Louis, Missouri, USA. Five blood films (accession numbers 47783-47787 NS, intensity of parasitemia is < 0.0001%, other data as for the type material) were deposited in the Institute of Ecology, Nature Research Centre, Vilnius, Lithuania.

DNA sequences: Mitochondrial cyt *b* lineage STGGAL1 with GenBank accession no. JN827318.

Site of infection: Mature erythrocytes; no other data.

Prevalence: Seven of 30 investigated swallow-tailed gulls (23.3%) were infected at the type locality.

Distribution and additional hosts: According to this study and the GenBank data, the lineage STGGAL1 and gametocytes of this parasite were recorded in eight swallow-tailed gulls (seven from the type locality and one from the island of Genovesa, Galapagos). This lineage was not reported from another seabird or land bird in Galapagos or elsewhere. The swallow-tailed gull breeds almost exclusively on the Galapagos Islands and therefore, the islands are the extent of the known distribution.

Etymology: This species is named in memory of Jenni Malie Higashiguchi, who was a graduate student at the University of Missouri – St. Louis (UMSL). Jenni was a bright and engaging colleague and a beloved friend of the campus community. Her research involved studying the hemosporidian parasites of the Galapagos Islands

through population studies of the potential mosquito vectors. Before coming to UMSL, she grew up and attended university in Hawaii, where she developed her love for birds and conservation biology. This species name is a tribute to her young life that ended while working so hard on the parasites of Galapagos birds.

Remarks

The most distinctive feature of development of *H. jenniae* is the presence of circumnuclear gametocytes occupying all cytoplasmic space in infected erythrocytes (Figs. 12, 16). Importantly, advanced growing gametocytes (Figs. 5-11, 13, 15), in which the pellicle is closely appressed to the erythrocyte envelope but does not extend to the erythrocyte nucleus, are common; this causes a 'cleft' and gives the gametocyte a markedly irregular appearance. Such 'clefts' have been recorded in growing gametocytes of many species of avian hemoproteids, but they are rare in circumnuclear or close to circumnuclear forms (see Figs. 10, 11). Fourteen *Haemoproteus* species with such gametocytes are known to parasitize birds (see Valkiūnas, 2005; Parsons et al., 2010): *H. archilochus, H. caprimulgi, H. circumnuclearis, H. fuscae, H. greineri, H. larae, H. pittae, H. platyleae, H. rotator, H. scolopaci, H. skuae, H. stableri, H. telfordi* and *H. velans. Haemoproteus jenniae* can be readily distinguished from these parasites, primarily due to the presence of large vacuole-like spaces in many growing gametocytes (Figs. 8, 13, 14).

Haemoproteus jenniae should be distinguished from *H. larae,* which produces similar gametocytes and parasitizes closely related species of the Laridae. To facilitate comparison of these parasites, the original microphotographs of *H. larae* from its type vertebrate host (black-headed gull) sampled at the type locality

(Southeast Kazakhstan) are given in Figs. 17-28 for the first time. *Haemoproteus larae* can be distinguished from *H. jenniae*, primarily due to 1) predominantly even outline of young gametocytes (compare Figs. 1-4 with Figs. 17-21), 2) compact macrogametocyte nuclei (compare Figs. 4, 11 with Figs. 20, 24), 3) readily distinguishable nucleoli (see Fig. 25), and 4) numerous oval and frequently even rod-like pigment granules (see Figs. 23, 26, 27). It is important to note that pigment granules in mature gametocytes of *H. larae* are markedly variable in shape and size, and oval-elongated granules predominate (see Figs. 25, 27); that is not a case in *H. jenniae* (see Figs. 6-12, 15) and is the most easily distinguishable difference between these 2 species. Additionally, fully-grown gametocytes of *H. jenniae* cause the marked hypertrophy of infected erythrocytes in width and the rounding up of the host cells, but that is not the case in fully-grown gametocytes of *H. larae* (compare Figs. 12 and 16 with Figs. 25 and 28, respectively).

Unfilled colorless spaces sometimes are visible in the infected erythrocytes with nearly mature gametocytes of *H. larae* before the gametocytes assume complete circumnuclear form (see Fig. 24). Such spaces are similar to vacuole-like spaces in gametocytes of *H. jenniae* (see Figs. 8, 13) and should be distinguished from them.

Phylogenetic relationships of parasites

Haemoproteus jenniae is clearly distinguishable in the phylogenetic tree (Fig. 29, clade B), which corresponds to its morphological features. Sequences of this parasite recovered from different individual hosts were identical, indicating lack of genetic diversity in this portion of the cyt *b* gene. The lineages of *H. jenniae* significantly cluster with lineages of hippoboscid-transmitted species of

Haemoproteus (Haemoproteus) spp., indicating that this parasite likely belongs to the subgenus *Haemoproteus.*

The genetic divergence among different lineages of readily morphologically distinguishable *H. jenniae,* and the hippoboscid-transmitted *Haemoproteus multipigmentatus* and *Haemoproteus columbae* (Fig. 29, clade B), ranges from 5.6-6.9% and 11-11.7%, respectively. Interestingly, the genetic distance in cyt *b* gene among closely related lineages of *H. jenniae* and *Haemoproteus iwa* is only 0.6% (Fig. 29); sequences of these morphologically readily distinguishable parasites differ only by 1 bp.

The genetic distance between *H. jenniae* and hemoproteids from the *Parahaemoproteus* clade (Fig. 29, clade A) ranges between 8.9% and 13.1%. Furthermore, the genetic distance among *H. jenniae* and *Haemoproteus* spp. reported in dolphin gull (*Larus scoresbii*) and black-tailed gull (*Larus crassirostris*) (Fig. 29, clade A) is 13.1% and 11.7%, respectively.

DISCUSSION

Haemoproteus jenniae was attributed to the subgenus *Haemoproteus* because cyt *b* lineages of this parasite cluster well with the lineages of the hippoboscid-transmitted species of hemoproteids, i.e., *H. multipigmentatus, H. columbae* and *H. iwa* belonging to the subgenus *Haemoproteus* (Fig. 29, clade B), but not to the lineages of the *Culicoides* spp.-transmitted hemoproteids belonging to the subgenus *Parahaemoproteus* (Fig. 29, clade A). Negligible genetic difference (0.6%) among cyt *b* sequences of *H. jenniae* and *H. iwa* is consistent with this conclusion. Hemoproteids of the subgenera *Parahaemoproteus* and *Haemoproteus* are transmitted

by species of Ceratopogonidae and Hippoboscidae, respectively. They undergo different modes of gametogenesis and sporogony in the vectors (Bennett et al., 1965; Atkinson, 1991; Valkiūnas, 2005) and, as a result, they usually fall in different clades in phylogenetic trees based on cyt *b* sequences. (Martinsen et al., 2008; Iezhova et al., 2010; Santiago-Alarcon et al., 2010; Valkiūnas, Santiago-Alarcon et al., 2010; Levin et al., 2011). It is probable that phylogenies based on this gene can be used for identification of subgenera of avian *Haemoproteus* (Iezhova et al., 2011). Vector species of *H. jenniae* need to be identified; the phylogenetic relationships of detected lineages (Fig. 29) suggest that hippoboscid flies should be investigated first.

In spite of the negligible genetic difference in cyt *b* sequences, *H. jenniae* and *H. iwa* are readily distinguishable based on morphology of their gametocytes. For instance, the number of pigment granules in macrogametocytes of *H. iwa* is at least twice that in microgametocytes; fully-grown gametocytes of this parasite are halteridial in shape; they do not assume circumnuclear form (Levin et al., 2011). These readily distinguishable features are not characteristic of *H. jenniae.* However, gametocytes of these two parasites also possess similarities: particularly in the morphology of their pigment granules and vacuolization of the cytoplasm (Levin et al., 2011). These data show how closely related and genetically similar lineages might belong to clearly different morphospecies, as is the case in *H. jenniae* and *H. iwa* (Fig. 29).

It is worth mentioning that lineages of unidentified *Haemoproteus* species (Fig. 29, clade A) were recorded in dolphin gull (*Larus scoresbii*) in Falkland Islands (Quillfeldt et al., 2010) and black-tailed gull (*Larus crassirostris*) in South Korea

(Ishtiaq et al., 2007). They clustered with lineages of *Culicoides* spp.-transmitted hemoproteids, such as *Haemoproteus lanii*, *H. passeris* and *H. balmorali* (Valkiūnas, 2005). Morphological description of these gull parasites is absent. Based on available phylogenetic information, it seems probable that hemoproteids of gulls might be transmitted by biting midges (Fig. 29, clade A) and hippoboscid flies (Fig. 29, clade B) and this warrants further investigation. This study and previously published data (Levin et al., 2011) indicate that the vertebrate host range should be carefully used in identification of subgenera of avian *Haemoproteus* because species of the subgenus *Haemoproteus* parasitize not only columbiform birds, as formerly believed, but also some species of marine birds.

We mainly used identified morphospecies of avian hemoproteids in the phylogenetic analysis (Fig. 29). Genetic distance among the great majority of cyt *b* lineages of readily distinguishable morphospecies is ≥ 5%. This is in accordance with the hypothesis of Hellgren et al. (2007) and recent data from Iezhova et al. (2011) that hemosporidian species with a genetic distance of ≥ 5% in the mitochondrial cyt *b* gene tend to be morphologically differentiated. However, this pattern certainly works only one direction; there are many readily distinguishable morphospecies with genetic divergence < 5% among their lineages, and as small as < 1% in some species, for instance *Haemoproteus minutus* and *Haemoproteus pallidus* (see Hellgren et al., 2007; Bensch et al., 2009; Valkiūnas et al., 2009; Iezhova et al., 2010). This is also the case with *H. jenniae* and *H. iwa*, which are the first examples of negligible genetic differences between readily distinguishable morphospecies from the clade of the subgenus *Haemoproteus* (Fig. 29, clade B). Additionally, these data indicate that

genetic distance information between lineages should be used carefully in understanding phylogenetic trees based on the cyt *b* gene; moreover, it can be used only in one direction. Mainly, the genetic distance of ≥ 5% in this gene testifies to probable morphological differentiation, but as small a difference as one nucleotide substitution might be present in morphologically well-differentiated parasites belonging both to *Haemoproteus* and *Parahaemoproteus* subgenera.

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Table I. Morphometry of host cells and mature gametocytes of *Haemoproteus jenniae* sp. nov. from the swallow-tailed gull *Creagrus furcatus*.

Feature	Measurements (μm) *
Uninfected erythrocyte	
Length	12.0-14.7 (13.3±0.7)
Width	6.4-7.3 (6.8±0.3)
Area	63.7-79.6 (72.8±4.0)
Uninfected erythrocyte nucleus	
Length	5.9-7.8 (6.7±0.5)
Width	2.2-2.9 (2.5±0.2)
Area	12.5-16.1 (14.1±1.0)
Macrogametocyte	
Infected erythrocyte	
Length	10.7-15.8 (13.1±1.2)
Width	7.0-9.8 (7.9±0.7)
Area	71.6-92.0 (81.1±5.1)
Infected erythrocyte nucleus	
Length	6.2-7.4 (6.6±0.3)
Width	1.9-3.0 (2.5±0.3)
Area	11.1-16.2 (14.0±1.3)

Gametocyte	
Length	18.7-26.1 (23.2±1.8)
Width	2.0-3.5 (2.8±0.4)
Area	46.2-68.8 (53.7±5.2)
Pigment granules	18.0-32.0 (25.0±4.4)
NDR†	0.6-1.0 (0.9±0.1)
Microgametocyte	
Infected erythrocyte	
Length	11.7-14.2(13.0±0.8)
Width	6.2-8.8 (7.8±0.8)
Area	69.8-90.4 (82.0±6.3)
Infected erythrocyte nucleus	
Length	6.0-7.2 (6.6±0.3)
Width	2.3-2.8 (2.5±0.2)
Area	12.6-15.8 (13.7±0.7)
Gametocyte	
Length	17.6-23.3 (20.4±1.8)
Width	2.1-3.4 (2.8±0.4)
Area	40.4-62.6 (51.3±7.9)
Pigment granules	13.0-28.0 (20.7±3.6)
NDR	0.5-1.0 (0.8±0.1)

Morphometry of macro- and microgametocyte nuclei is not given due to markedly diffuse structure of the nuclei and difficulty to measure them.

* All measurements (n=21) are given in micrometers. Minimum and maximum values are provided, followed in parentheses by the arithmetic mean and standard deviation.

† NDR = nucleus displacement ratio according to Bennett and Campbell (1972).

FIGURE LEGENDS

FIGURES 1-16. *Haemoproteus jenniae* sp. nov. from the blood of swallow-tailed gull *Creagrus furcatus*. (**1-4**) Young gametocytes. (**5-12**) Macrogametocytes. (**13-16**) Microgametocytes. Long simple arrows – nuclei of parasites. Short simple arrows – pigment granules. Triangle arrow heads – vacuole-like spaces. Giemsa-stained thin blood films. Bar = 10 μm.

FIGURES 17-28. *Haemoproteus larae* from the blood of black-headed gull *Chroicocephalus ridibundus*. (**17-21**) Young gametocytes. (**22-25**) Macrogametocytes. (**26-28**) Microgametocytes. Long simple arrows – nuclei of parasites. Long triangle arrow – nucleolus. Short simple arrows – pigment granules. Simple arrow head – unfilled colorless space visible in the infected erythrocyte (**24**); such spaces are similar to vacuole-like spaces in gametocytes of *H. jenniae* (see Figs. 8, 13) and should be distinguished from them. Giemsa-stained thin blood films. Bar = 10 μm.

FIGURE 29. Maximum likelihood (ML) phylogenetic hypothesis of avian *Haemoproteus* parasites based on approximately 550 bp of the mitochondrial cytochrome *b* gene. Two lineages of *Plasmodium* species are used as outgroups. GenBank accession numbers are given after parasite species names, with the names of new species in bold. ML bootstrap values greater than or equal to 80 are indicated near the nodes. Vertical bars indicate group of closely related lineages of hemoproteids belonging to the subgenera *Parahaemoproteus* (clade A) and *Haemoproteus* (clade B).

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Figure 1-16:

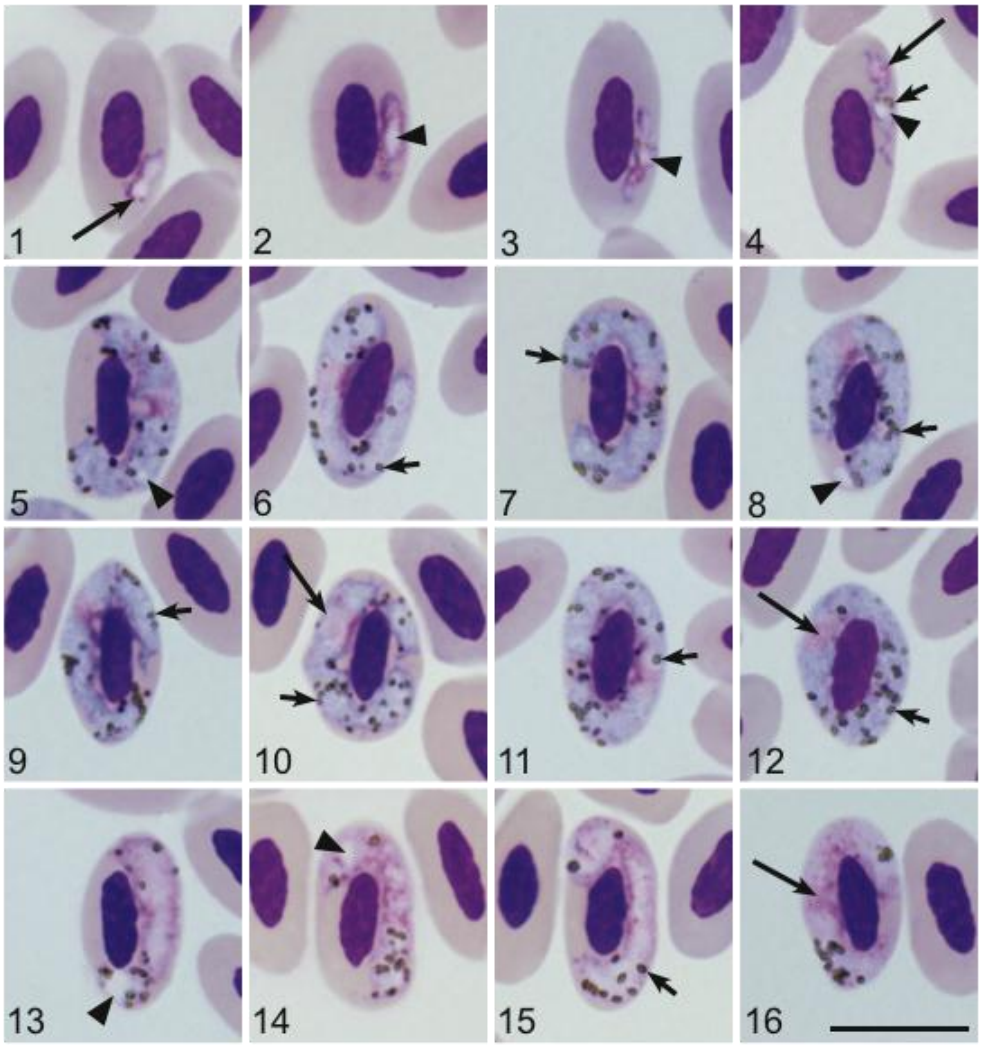


Figure 17-28:

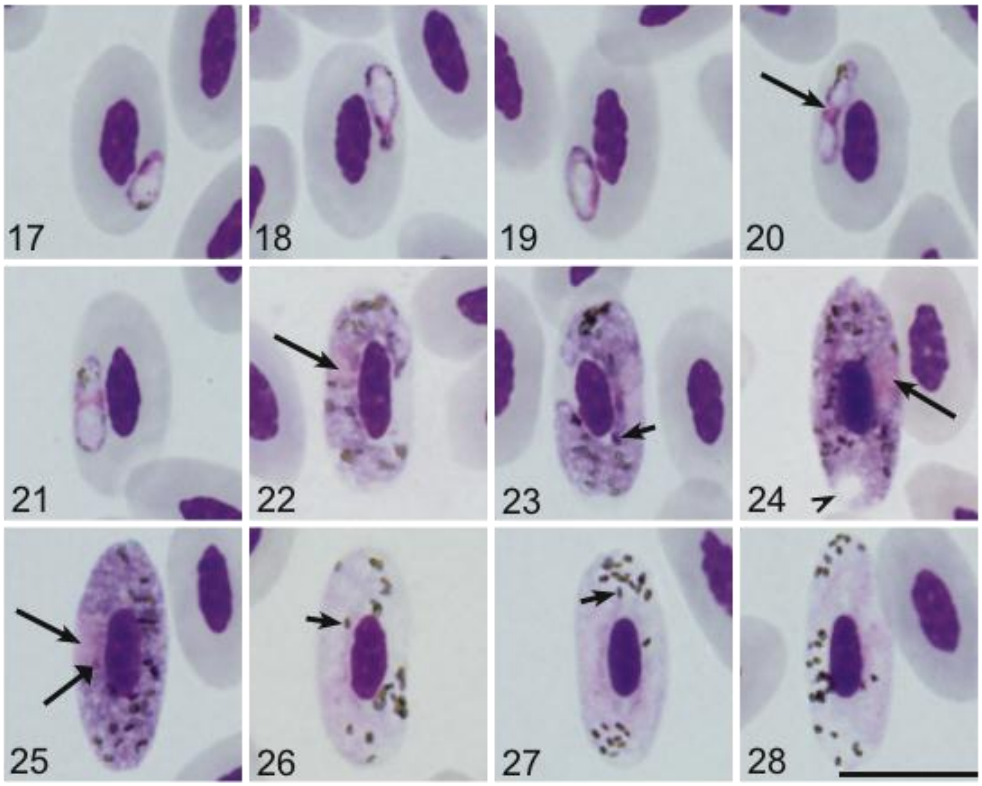
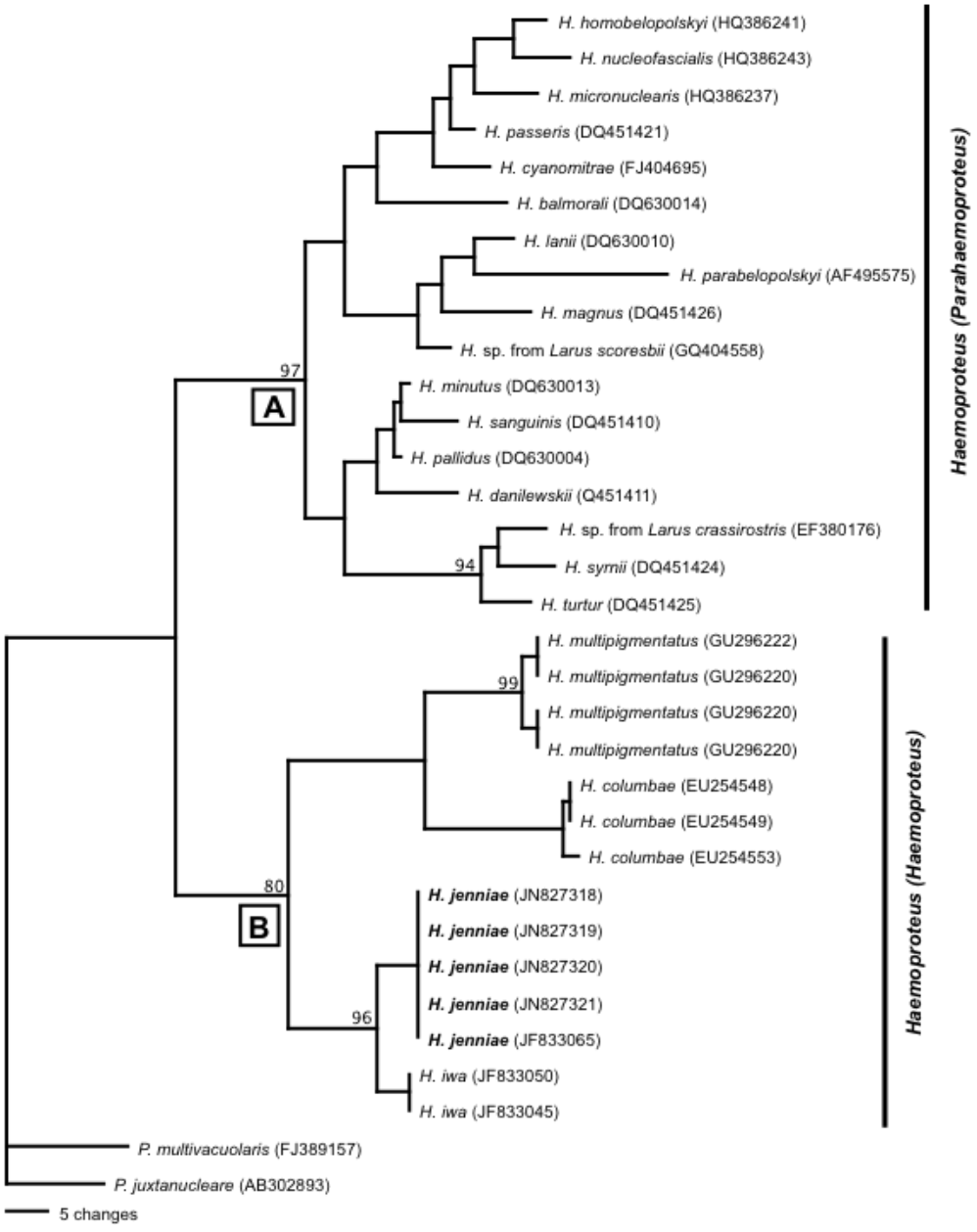


Figure 29:


H. homobelopolskyi (HQ386241)
H. nucleofascialis (HQ386243)
H. micronuclearis (HQ386237)
H. passeris (DQ451421)
H. cyanomitrae (FJ404695)
H. balmorali (DQ630014)
H. lanii (DQ630010)
H. parabelopolskyi (AF495575)
H. magnus (DQ451426)
H. sp. from Larus scoresbii (GQ404558)
97
A
H. minutus (DQ630013)
H. sanguinis (DQ451410)
H. pallidus (DQ630004)
H. danilewskii (Q451411)
H. sp. from Larus crassirostris (EF380176)
94
H. symii (DQ451424)
H. turtur (DQ451425)
Haemoproteus (Parahaemoproteus)
H. multipigmentatus (GU296222)
99
H. multipigmentatus (GU296220)
H. multipigmentatus (GU296220)
H. multipigmentatus (GU296220)
H. columbae (EU254548)
H. columbae (EU254549)
H. columbae (EU254553)
80
B
H. jenniae (JN827318)
H. jenniae (JN827319)
H. jenniae (JN827320)
H. jenniae (JN827321)
96
H. jenniae (JF833065)
H. iwa (JF833050)
H. iwa (JF833045)
Haemoproteus (Haemoproteus)
P. multivacuolaris (FJ389157)
P. juxtanucleare (AB302893)
5 changes

Chapter IV: Long-term isolation of a highly mobile seabird on the Galapagos

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Abstract: The Galapagos Islands are renowned for their high degree of endemism. Marine taxa inhabiting the archipelago might be expected to be an exception, because of their utilization of pelagic habitats - the dispersal barrier for terrestrial taxa - as foraging grounds. Magnificent frigatebirds (*Fregata magnificens*) have a highly vagile lifestyle and wide geographical distribution around the South and Central American coasts. Given the potentially high levels of gene flow among populations, the species provides a good test of the effectiveness of the Galapagos ecosystem in isolating populations of highly dispersive marine species. We studied patterns of genetic (mitochondrial DNA, microsatellites and nuclear introns) and morphological variation across the distribution of magnificent frigatebirds. Concordant with predictions from life-history traits, we found signatures of extensive gene flow over most of the range, even across the Isthmus of Panama, which is a major barrier to gene flow in other tropical seabirds. In contrast, individuals from the Galapagos were strongly differentiated from all conspecifics, and have probably been isolated for several hundred thousand years. Our finding is a powerful testimony to the evolutionary uniqueness of the taxa inhabiting the Galapagos archipelago and its associated marine ecosystems.

Introduction

Darwin was strongly influenced by the uniqueness of many Galapagos taxa when he conceived On the Origin of Species [1]. He hypothesized that many

Galapagos endemics arose from in situ radiations, following initial colonization of the archipelago by ancestral species. For numerous taxa, this view has received support from morphological and molecular studies (reviewed in [2]). However, Darwin noted that '...it is obvious that marine birds could arrive at these (Galapagos) islands much more easily and frequently than land-birds...', and thus show a much lower degree of endemism ([1], p. 348). Indeed, while all native reptiles and terrestrial mammals and 84 percent of terrestrial birds are endemic [3], only 37 percent (7 out of 19) of Galapagos seabird species are currently classified as endemic. Because seabirds and other marine species forage in the pelagic zone, which is the isolating agent for terrestrial species, the 1000 km of open ocean separating the Galapagos archipelago from the mainland could link archipelago to continental populations, especially in highly dispersive species.

Species predicted to be least susceptible to isolation effects on the Galapagos would be far-ranging in the pelagic zone, and habitat generalists with a widespread occurrence in the surrounding coastal and marine environments of South and Central America. Such species residing on the Galapagos would encounter suitable habitat should they disperse back to the mainland. Further, in species exhibiting gene flow across large geographical distances, one would predict recurrent arrival of immigrants to the Galapagos, counteracting allopatry and potentially swamping out local adaptation.

Some of the endemic seabird taxa of the Galapagos Islands have no flight capabilities (e.g., Galapagos penguin, flightless cormorant). The most capable flyers among seabirds that breed on the Galapagos are probably the albatrosses

and frigatebirds. Albatrosses perform long-distance foraging trips [4] and most albatross species exhibit extensive gene flow across vast geographical distances [5]. However, weak prevailing winds around the inner tropical convergence zone are thought to restrict the flight patterns of albatrosses, which have relatively high wing loading, or relatively small wings for their body weight [6,7]. Indeed, only four albatross species occur outside the Southern Hemisphere oceans, and their ranges are very restricted, including that of the Galapagos-endemic waved albatross (*Phoebastria irrorata*).

Magnificent frigatebirds are perhaps the least likely of Galapagos species to be subject to geographical isolation. These tropical seabirds are widely distributed along the Atlantic and Pacific coasts of Central and South America, and on neighbouring archipelagos, including the Galapagos. They are observed as vagrants far north along the eastern and western coasts of North America, and have even reached western Europe and Africa, usually after big storms [8]. The species has the lowest wing loading (i.e. smallest body mass relative to the area of its wings [9]) among birds and is known for its soaring behaviour. It uses thermal winds to reach high altitudes, and can travel hundreds of kilometres at slow speed, even while tending an active nest [9]. This combination of life- history traits makes the magnificent frigatebird especially suitable for studying gene flow and isolation in highly mobile species of the Galapagos.

Here we present data from three classes of genetic markers (mitochondrial DNA, microsatellites and nuclear introns) surveyed in magnificent frigatebirds from across their distribution. The markers reflect both (i) maternally and biparentally

inherited lineages and (ii) rapidly and slowly evolving genomic regions, providing a comprehensive view of genetic differentiation. We also provide morphological data that enable us to investigate patterns of phenotypic differentiation within the species, and how they relate to the patterns of genetic variation. Based on widespread sampling across the species's distribution range, we investigate whether gene flow among non-Galapagos colonies is extensive. We then determine whether geographical structuring of genetic and morphological variation supports or rejects a scenario of allopatric isolation of magnificent frigatebirds on the Galapagos.

MATERIALS AND METHODS

Sampling

We sampled 232 individuals from nine populations across the range of the magnificent frigatebird (tables 1, 2 and figure 1), including 221 fresh samples and 11 samples from toe-pads of museum specimens collected between 1895 and 1986 (electronic supplementary material, table S1). We collected fresh blood or plucked feathers from nestlings or adults on active nests, ensuring that resident birds were sampled. Birds were individually marked during sampling, and we did not sample offspring and adults from the same nest. Samples are therefore presumably unrelated, at least with regard to the present generation. Blood samples were stored in lysis buffer and frozen once in the laboratory. Toe-pad samples were from Pacific localities, extending our sampling in a geographical region otherwise covered only by Galapagos and Panamanian samples. Very small pieces of toe-pads were cut from the museum specimens using clean scalpel blades and stored dry until extraction.

Laboratory methods

Following digestion with Proteinase K, DNA was extracted from modern samples using standard phenol – chloroform, salt precipitation or Qiagen kit (Qiagen, Valencia, USA) methods. DNA from museum toe-pads was extracted in a facility solely dedicated to 'ancient' DNA work. We followed stringent protocols to avoid and detect potential contamination (see [10,11]).

Mitochondrial DNA

We amplified fragments of three genes, ATP6 (531 base pairs (bp)), cytochrome *b* (550 bp) and ND2 (555 bp; sequence lengths do not include the primers). Details of the PCRs are given in the electronic supplementary material. All PCRs of museum material were set up in an 'ancient' DNA laboratory, and negative and positive controls were used throughout (details in the electronic supplementary material). PCR products were cleaned using EXOSAP (USB Scientific, Cleveland, USA). Both strands of DNA were cycle-sequenced with the PCR primers using BIGDYE v. 3.1 (Applied Biosystems, Foster City, USA), followed by an ethanol or Sephadex clean-up. Sequences were run on an ABI 3130xl instrument and assembled in SEQUENCHER v. 4.8 (Gene Codes, Ann Arbor, USA).

Microsatellite markers

Following initial assessment of multiple microsatellite markers (see electronic supplementary material), we selected eight loci that exhibited multiple alleles, showed reliable amplification and could be scored consistently: Fmin02, Fmin11, Fmin12, Fmin14, Fmin15, Fmin16, Fmin17 and Fmin18 [12]. The loci were amplified in three multiplex PCR reactions using fluorescently labeled forward

primers (electronic supplementary material, table S3) and run on an ABI 3130xl instrument. Genotypes were scored in GENEMAPPER v. 4.0.

Nuclear introns

For a subset of samples (electronic supplementary material, table S4) we amplified four introns [13,14] from the nuclear genes a-enolase (ENOL), glyceraldehyde-3-phosphate dehydrogenase (GAPD), myelin proteolipid protein (MPP) and ornithine decarboxylase (OD), in total 1595 bp. PCR products were cleaned and sequenced on both strands as described above. Intron sequences heterozygous for indels were analysed and phased using CHAMPURU [15] and INDELLIGENT [16]. All sequences obtained in this study have been submitted to the GenBank database (accession numbers: FR691079 – FR691320).

Data analysis

To visualize the genealogical relationships among haplotypes, we generated statistical parsimony networks of mitochondrial and nuclear sequences using TCS [17]. For evolutionary calculations based on mitochondrial DNA (mtDNA) and whenever implemented in the software, we chose the HKY model of sequence evolution; transition – transversion ratio was set to 47, as estimated using the AIC test in JMODELTEST v. 0.1.1 [18]. Otherwise, we used the next simplest model available, which at divergence levels below1 percent (see §3) has only a minor effect on the outcome. Standard nuclear diversity indices (haplotype and nucleotide diversity) were calculated in DNASP v. 5 [19] and ARLEQUIN v.3.5.1.2 [20]. The mean net nucleotide distance among groups was calculated in MEGA v.4.1 [21] using

the K2P model; standard errors were estimated based on 1000 bootstrap replicates across sites.

GENEPOP on the web (http://genepop.curtin.edu.au/) was used for standard population genetic data quality assessment tests, including tests for heterozygote deficit/excess and linkage disequilibrium, applying sequential Bonferroni correction. To account for differences in sample size among locations, we calculated the rarified mean number of alleles per locus using HP-RARE [22]. Principal coordinates analysis (PCA) of individual genotypes was performed in GENALEX [23]; F-statistics were calculated in GENETIX [24]. The latter provide a measure of genetic differentiation (fixation index) that quantifies the genetic distance among populations, with larger values indicating higher differentiation. Assignment tests based on multi-locus microsatellite genotypes were performed in GENECLASS v.2.0 [25] using the Bayesian algorithm of Rannala & Mountain [26], and the same data were evaluated in a Bayesian genotype clustering procedure in STRUCTURE v.2.3.3 [27]. We employed default settings in the newly implemented *Locprior* model [28], which is designed for cases of especially weak population structure, and assumed correlated allele frequencies. For each value of K (number of demes assumed for the clustering procedure), we performed two long runs of 500 000 iterations each (after a burn-in of 200 000 steps) and averaged the results. Multiple additional shorter runs were performed using different settings (admixture model, no-admixture model) to check for convergence and to assess the importance of model choice.

The three datasets were analysed separately using a Bayesian coalescent-based framework in MIGRATE v. 3.0.7 [29,30], a procedure that jointly estimates Q

(a measure of effective population size) and unidirectional migration rates among populations. To limit the number of parameters to be estimated, we grouped all samples a priori into three geographical regions (Galapagos, eastern Pacific, Atlantic). Runs were initiated based on starting values from FST values and used wide uniform priors. Multiple additional runs were performed using results from earlier runs as starting conditions, still using flat priors but longer chains (see electronic supplementary material, table S5 for details).

To estimate the mtDNA phylogeny and to date the ages of the splits among main clades, we employed the Bayesian- relaxed (uncorrelated lognormal) molecular clock approach implemented in the program BEAST v.1.5.3 [31]. Trees were rooted with the sister taxon Fregata aquila (GenBank accession numbers EU166963, EU166990, AY369064 [32]). Settings included a Yule prior to model lineage birth, a normal distribution of substitution rate (mean 2.13 + 0.065% divergence per million years; see [33]). We also calibrated the tree using an assumed maximum age of separation from the sister taxon F. aquila, of 1 Myr, based on geological dating of the emergence of Ascension Island [34]. BEAST analyses were run for up to 300 million generations, and convergence was checked in TRACER v.1.5 (available from http://beast.bio.ed.ac.uk/Tracer) and by comparing results from independent runs.

Morphological measurements

We collected a series of morphometric measurements from specimens in museum collections (electronic supplementary material,table S6).We measured wing (length of the unflattened first primary), inner tail and outer tail (innermost and outermost tail feather, respectively) culmen length (starting at the end of feather cover

at the bill origin), bill depth and bill width (measured at the starting point of culmen), and the length of the middle toe (taken from the end of the skin towards the claw, to the third joint counting from the claw; electronic supplementary material, figure S2). All measurements were recorded to the nearest millimetre using a calliper, except for wing length, which was measured to the nearest 0.5 mm using a ruler. All measurements were taken by the same person (F.H.), using five males and five female individuals from the Galapagos (roughly two-thirds of all Galapagos specimens available in US museums). For comparison, we measured 16 males and 11 female museum specimens from eastern Pacific and Atlantic locations. Body size measurements were compared statistically using U-tests in R [35]. R was also used to perform linear discriminant function analysis, following log-transformation of all measurements.

RESULTS

Basic information and statistics on the variability of the employed markers are given in the electronic supplemental material.

Population genetic structure

Mitochondrial DNA

A statistical parsimony network of mtDNA sequences (figure 2) showed a deep split into two main lineages, separated by 14 nucleotide changes, or a mean net sequence divergence of 0.88 ± 0.24% (s.e.; same result for Kimura two-parameter and Tamura-Nei model distances). One lineage consisted of individuals from the Atlantic and eastern Pacific populations (together referred to as ‘non- Galapagos’), while the second lineage was confined to the Galapagos (electronic supplementary material,

tables S7 and S8). Consistent with its wider geographical distribution, the former lineage harboured more genetic diversity (33 haplotypes, $\pi = 0.00126 \pm 0.00006$) than the Galapagos lineage (three haplotypes, $\pi = 0.00012 \pm 0.00018$). Pairwise ϕ_{ST} values among localities (electronic supplementary material, table S9) confirmed this finding: all comparisons between Galapagos and non-Galapagos populations were larger than 0.90 and statistically significant. In contrast, all comparisons among non-Galapagos populations yielded FST values smaller than 0.20; most of these were non-significant, even between ocean basins.

Non-Galapagos birds exhibited extensive haplotype sharing among populations (figure 2). The two most frequent haplotypes (BMF01, BMF06) were present in every sampled population except the Galapagos, and found in almost 60 percent of those individuals. Frequent haplotypes were shared among eastern Pacific and Atlantic populations, and only rare haplotypes were confined to one or two populations.

A relaxed molecular clock model in BEAST indicated that the Galapagos and non-Galapagos lineages diverged several hundred thousand years ago. The geometric mean of the posterior distribution was 247 200 years before present (YBP), and the 95 percent higher posterior density spanned 82 800 – 657 400 YBP. Despite the potential drawbacks associated with divergence dating based on mtDNA [36], this indicates with high certainty that the two lineages split during the Middle or Late Pleistocene, well before the last glacial maximum (around 22 000 YBP).

Microsatellites

Genetic diversity within populations was relatively similar among sampling locations, except for the less variable Galapagos population (table 2). As for mtDNA, analyses of population structure recovered two strongly differentiated main groups. PCA clearly separated the Galapagos samples from all others (figure 3). Non-Galapagos genotypes showed little or no geographical structuring, even between ocean basins: eastern Pacific and Atlantic individuals overlapped almost completely in the PCA, and STRUCTURE did not provide any additional resolution (electronic supplementary material, figure S1). Similarly, all pairwise F_{ST} values involving the Galapagos were larger than 0.34 and significant, while the remaining values were smaller than 0.05 and non- significant in all but three cases, including most cross-isthmus comparisons (electronic supplementary material, table S10). An assignment test in GENECLASS provided perfect resolution between Galapagos and non-Galapagos samples, but poor resolution among the non-Galapagos populations (electronic supplementary material, table S11).

Nuclear intron markers

Assessment of haplotypes (figure 4 and electronic supplementary material, table S4) revealed a diagnostic character at the OD locus, separating the Galapagos from all other individuals. Large and significant frequency differences between Galapagos and all other samples were found at GAPD and ENOL.

For all three marker systems, Bayesian coalescent simulations in MIGRATE indicated a much lower Q (effective population size) value for the Galapagos than for non- Galapagos populations, and suggested the absence of gene flow among

Galapagos and continental populations (mode at zero), despite wide posterior credibility intervals. No gene flow was indicated in an eastward direction across the isthmus by all marker systems, but analyses of mitochondrial and microsatellite data indicated significant westward gene flow from Atlantic into eastern Pacific populations. The posterior distributions for all migration estimates had a clear maximum at zero, except the estimate from Atlantic into the eastern (non- Galapagos) Pacific, which showed a peak at 25 (mtDNA) and 433 (microsatellites). Demographic analyses (electronic supplementary material, tables S12 and S13) indicated pronounced recent population growth of Galapagos as well as non-Galapagos lineages.

Morphological measurements of museum specimens

Three to four size measurements (depending on the sex) indicated that Galapagos birds were significantly larger than those from the mainland ($p < 0.05$; table 3). Those measurements included wing, inner tail and outer tail (both sexes), and culmen (females only). A multivariate discriminant function analysis performed separately for males and females correctly classified 100 per cent of individuals to their region of origin (Galapagos or non-Galapagos), and a subsequent leave-one-out cross-evaluation procedure classified about 80 per cent of individuals correctly. The latter may relate to our limited sample size, or indicate only subtle inter-regional differences at the surveyed morphometric characters.

DISCUSSION

All marker types indicated extensive gene flow across most of the range of the magnificent frigatebird, but pronounced population structure separating the

Galapagos from all other populations. This signal was also reflected in significant morphological differences between Galapagos and mainland birds. The Galapagos archipelago has long received attention for its high degree of endemism and has been recognized as a showcase for evolutionary processes (e.g. [2]). A new case documenting endemism on the Galapagos is thus not surprising per se. However, the behaviour and ecology of magnificent frigatebirds render them one of the least likely of Galapagos taxa to have evolved in isolation from its conspecifics.

Magnificent frigatebirds are renowned for their wide-ranging behaviour [9]. Finding little or no genetic structure among continental populations, despite the use of high-resolution genetic markers, is consistent with this high dispersal capability. Importantly, our results reveal signatures, at all three classes of genetic markers, of extensive gene flow even between Atlantic and Pacific colonies. This is consistent with field observations ([37]; Frank Hailer 2007, personal observation). The Isthmus of Panama closed approximately 2.8 Myr ago and has since posed a major barrier to gene flow in numerous marine species [38,39], including highly dispersive taxa (e.g. [40]). To our knowledge, the magnificent frigatebird is thus the first tropical seabird for which extensive natural gene flow across the Isthmus of Panama has been suggested.

Explanations for the uniqueness of magnificent frigatebirds on the Galapagos

Many seabirds show pronounced natal and breeding philopatry (i.e. a tendency to return to breed at the location they were born or had bred previously). Long-term field data are lacking for magnificent frigatebirds, but short- term data suggest some degree of philopatry also in this species [8]. The ultimate causes for

such philopatry are not known. Among several factors, familiarity with natal and/or previous breeding habitats has been suggested as a driver of philopatry [41]. However, the inherent contrast in our findings between the Galapagos and the non-Galapagos range suggests that a factor unique to the Galapagos population may be promoting evolutionary isolation on the archipelago. One potential mechanism is the presence of some barrier to movement between the Galapagos and the mainland [42]. Alternatively, a behavioural mechanism related to the elaborate courtship rituals of frigatebirds [8] could be causing allopatric isolation.

The Galapagos archipelago is located approximately 1000 km from the South American mainland. Galapagos seabirds have been reported to forage predominantly to the west of the archipelago, attracted by local upwelling of cold, nutrient-rich waters that lead to higher prey availability [43]. Seabirds from the South American mainland, however, tend to forage in the nearby and highly productive upwelling zone along the continental shelf [41], so many of them may not venture out far from the coast. A recent review of seabird population structuring [42] found that most populations occupying separate ranges during the non-breeding season also display population genetic structure. Our results regarding the Galapagos population could thus be explained by geographical/foraging range isolation. For instance, magnificent frigatebirds could be avoiding dispersal across the open ocean, despite their far-ranging behaviour [9], and despite our genetic results from the non-Galapagos lineage. Extensive dispersal in the non-Galapagos range under this scenario might be oriented along coastlines and among more proximate islands [44].

However, magnificent frigatebirds banded in Galapagos have been recovered as dead and/or emaciated vagrants in Central America (Carlos Valle, Galapagos Academic Institute for the Arts and Sciences 2010, personal communication), demonstrating movement of individuals across the potential barrier. Similarly, recent data from frigatebird *Haemoproteus* blood parasites suggest that there may be physical interactions between Galapagos and continental frigatebirds (Levin et al., unpublished data). In the Nazca booby (*Sula granti*), banding records have demonstrated reproduction of Galapagos-banded individuals on the mainland [45]. Surprisingly, and in contrast to this movement data, our results indicate long-term isolation on the Galapagos, probably for several hundred thousand years. Over those time frames, the global climate has changed cyclically, with marked fluctuations of trade wind patterns [46], water nutrient levels [47], sea level [48], sea surface temperature [49] and circulation patterns [50], implying vast changes to marine habitats. Tropical seabirds have thus experienced significant spatio-temporal fluctuations of the available marine nutrients (and thus of their prey), which probably influenced their foraging patterns. Given their capacity or long-distance flight, magnificent frigatebirds have had ample opportunity to move between the Galapagos and the continent, calling for consideration of adaptive scenarios to explain the lack of gene flow between those regions.

Magnificent frigatebirds and great frigatebirds *F. minor* occur in sympatry on the Galapagos. Typically, only one of the two frigatebird species is found breeding at a given location (but see [51] for another rare, and possibly recent [52], instance of sympatry between those species). If interspecific hybridization is disadvantageous,

selection should favour behavioural avoidance of mating between magnificent and great frigatebirds. While very rare hybridization between the two species has been anecdotally reported, such field observations are difficult because of the complex plumage maturation patterns of frigatebirds (Carlos Valle 2010, personal communication; [8]). Genetic data from Galapagos great frigatebirds lack signals of introgression and thus indicate reproductive isolation (Hailer et al., unpublished data). As a by-product of increased selectiveness for mates, magnificent frigatebirds on the Galapagos may thus reject their conspecifics from the mainland (i.e. character displace- ment). More data on individual movement and mechanisms of mate choice in frigatebirds on the Galapagos are necessary to evaluate this hypothesis. Future studies may reveal the exact mechanism of how such a highly dispersive species maintains long-term genetic differentiation on the Galapagos.

The evolutionary distinctiveness of the Galapagos population of the magnificent frigatebird necessitates separate management. This population encompasses approximately 1000 pairs, distributed across four islands [53]. Possible catastrophic events, along with recent human impacts, could seriously threaten its survival, especially during El Niño years, which are associated with dramatic population size reductions in Pacific seabirds [54]. Current classification of the Galapagos population as Least Concern [55] should therefore be revisited.

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Figures and Table captions

Table 1: Genetic variation in Magnificent Frigatebird populations across three mtDNA regions (n denotes sample size, NH number of unique haplotypes, and HD and π are gene and haplotype diversities, respectively). Belize populations are HC (Halfmoon Caye) and MW (Man O'War Caye).

Table 2: Genetic variability in Magnificent Frigatebird populations at eight microsatellite markers. n denotes sample size (number of individuals), AR rarefied allelic richness (Kalinowski, 2005), HE and HO are unbiased expected and observed heterozygosity, respectively.

Table 3: Morphometric measurements of Magnificent Frigatebird museum specimens. Numbers given are mean±S.D. Significant differences within sexes among regions are marked by asterisks ($p<0.01$, U test).

Figure 1: Sampling locations and sample sizes of Magnificent Frigatebirds analyzed in this study. Small yellow dots denote toe-pad samples.
BMF – Bahamas, BVI – British Virgin Islands, CY – Little Cayman, DT – Dry Tortugas (Florida, USA), Gal – Galapagos (Ecuador), HC – Halfmoon Caye (Belize), IG – Isla Iguana (Panama), Jam - Jamaica, MW – Man O'War Caye (Belize).

Figure 2: Statistical parsimony network of mtDNA sequences (1636 basepairs). Pie charts and filled circles correspond to haplotypes, circle area being proportional to their frequency. Inferred intermediate steps are shown as small open circles, dotted lines are less likely genealogical pathways (based on haplotype frequencies). Haplotypes are named as in Table S7.

Figure 3: Principal coordinates analysis of microsatellite genotypes. Symbols denote individuals, with their multilocus genetic ancestry scaled on two axes.

Figure 4: Statistical parsimony networks of sequence variation in nuclear introns. Pie charts and filled circles denote haplotypes, black dots are inferred intermediate steps. For clarity, the four Atlantic populations are all shown in white (see table S4).

Table 1

Region	Population	n	N_H	HD±SD	π±SD
Galapagos	**North Seymour**	**20**	**3**	**0.195±0.115**	**0.00012±0.00007**
Eastern Pacific	**(overall)**	**36**	**11**	**0.867+-0.031**	**0.00143+-0.00089**
	Panama	*25*	*9*	*0.863±0.040*	*0.00128±0.00012*
	toe-pads	*11*	*8*	*0.927±0.066*	*0.00187±0.00037*
Atlantic	**(overall)**	**175**	**26**	**0.760+-0.030**	**0.00121+-0.00076**
	Bahamas	*29*	*5*	*0.421±0.110*	*0.00076±0.00020*
	Florida	*29*	*8*	*0.675±0.087*	*0.00104±0.00019*
	Brit. Virgin Isl.	*21*	*12*	*0.852±0.071*	*0.00133±0.00018*
	Jamaica	*30*	*10*	*0.897±0.027*	*0.00152±0.00009*
	Cayman Isl.	*30*	*9*	*0.786±0.0065*	*0.00135±0.00017*
	Belize (HC)	*13*	*5*	*0.795±0.076*	*0.00111±0.00014*
	Belize (MW)	*23*	*6*	*0.708±0.090*	*0.00089±0.00016*

Table 2

Region	Population	n	AR	HE±SD	HO±SD
Galapagos	*North Seymour Isl.*	*20*	*4.6*	*0.54±0.11*	*0.58±0.04*
Eastern Pacific	*Panama*	*25*	*5.6*	*0.62±0.09*	*0.61±0.04*
Caribbean	*Bahamas*	*29*	*6.3*	*0.68±0.09*	*0.69±0.03*
	Florida	*29*	*6.0*	*0.68±0.08*	*0.68±0.03*
	British Virgin Isl.	*21*	*6.0*	*0.65±0.09*	*0.69±0.04*
	Jamaica	*28*	*5.9*	*0.65±0.09*	*0.67±0.03*
	Cayman Isl.	*30*	*5.6*	*0.65±0.09*	*0.65±0.03*
	Belize HC	*13*	*6.0*	*0.66±0.09*	*0.65±0.05*
	Belize MW	*24*	*5.7*	*0.63±0.09*	*0.58±0.04*

Table 3

	wing (cm)	outer Tail (cm)	inner Tail (cm)	culmen (mm)	bill depth (mm)	bill width (mm)	middle toe (mm)
males							
Galapagos (n=5)	64.0±0.9 *	49.1±2.2 *	21.8±1.3 *	109.6±4.2	30.2±1.5	29.8±1.9	42.0±2.0
non-Galapagos (n=16)	61.8±1.3	45.8±3.3	18.2±1.3	107.5±3.3	28.9±1.2	29.3±1.2	41.1±1.1
females							
Galapagos (n=5)	68.8±0.8 *	54.7±1.5 *	22.1±3.4 *	125.2±2.2 *	32.4±1.1	31.2±0.8	43.8±0.4
non-Galapagos (n=11)	64.7±1.2	47.4±2.1	18.0±0.5	119.8±3.1	31.7±1.6	32.2±1.2	43.7±0.8

Figure 1

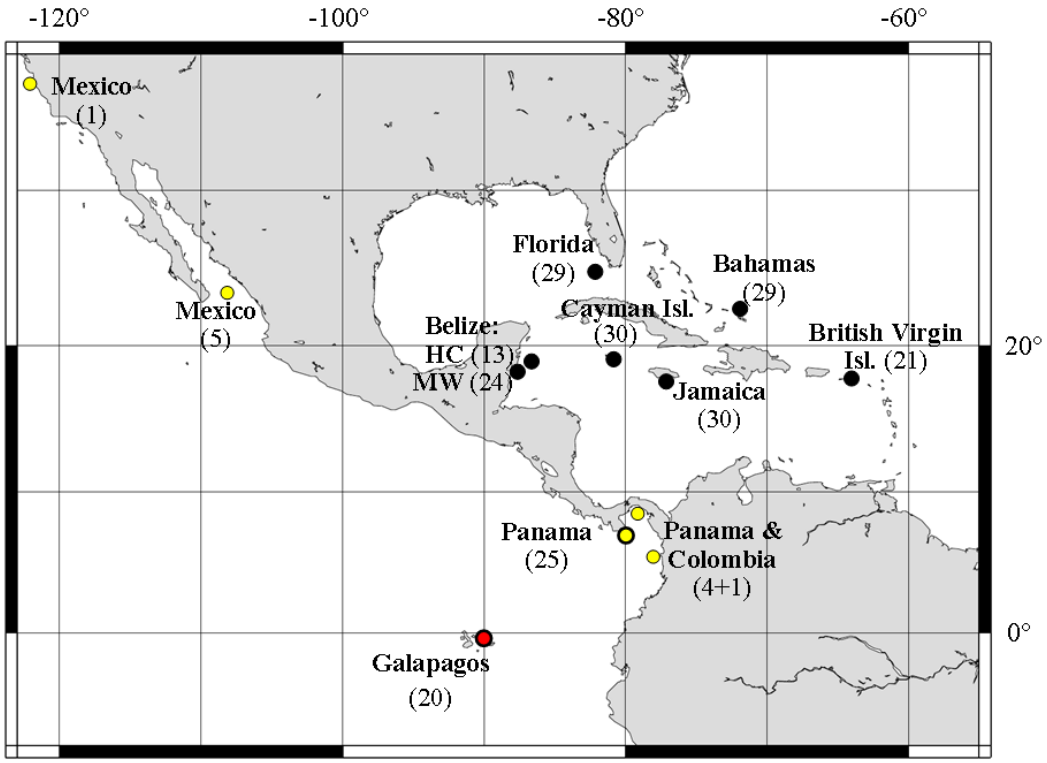

-120°
-100°
-80°
-60°
20°
0°
Mexico
(1)
Mexico
(5)
Florida
(29)
Bahamas
(29)
Cayman Isl.
(30)
Belize:
HC (13)
MW (24)
Jamaica
(30)
British Virgin
Isl. (21)
Panama
(25)
Panama &
Colombia
(4+1)
Galapagos
(20)

Figure 2

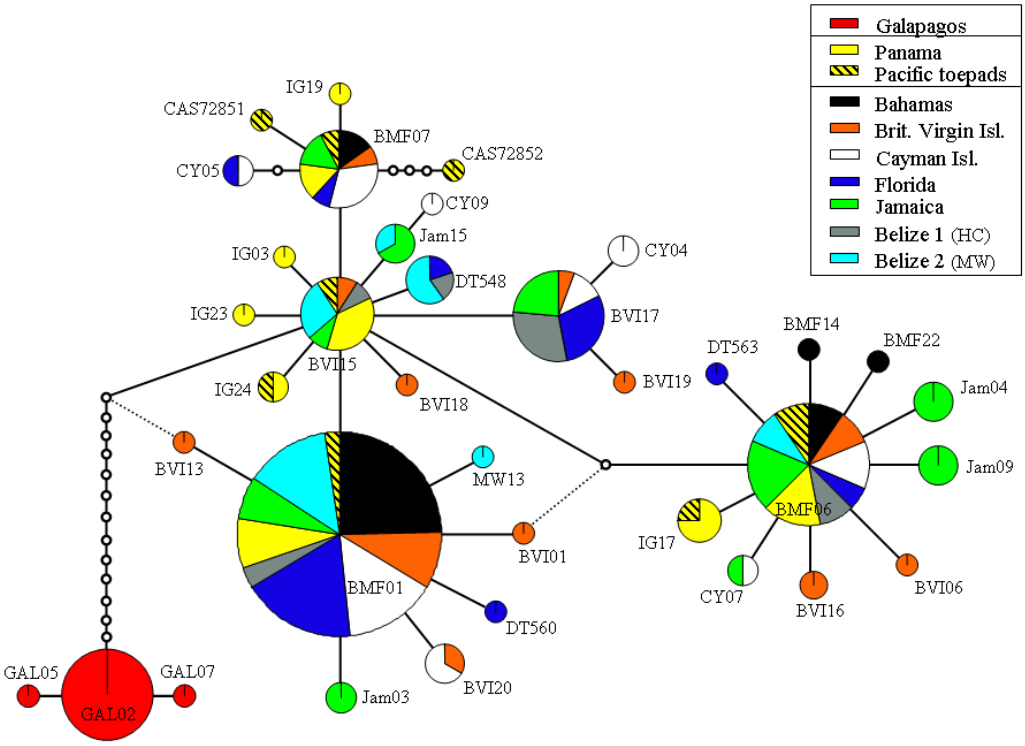

Galapagos
Panama
Pacific toepads
Bahamas
Brit. Virgin Isl.
Cayman Isl.
Florida
Jamaica
Belize 1 (HC)
Belize 2 (MW)
IG19
CAS72851
BMF07
CAS72852
CY05
CY09
Jam15
IG03
DT548
CY04
IG23
BVI15
BVI17
IG24
BVI18
BVI19
BMF14
DT563
BMF22
Jam04
BVI13
MW13
Jam09
BMF06
IG17
BVI01
BMF01
DT560
CY07
BVI16
BVI06
BVI20
GAL05
GAL07
GAL02
Jam03

Figure 3

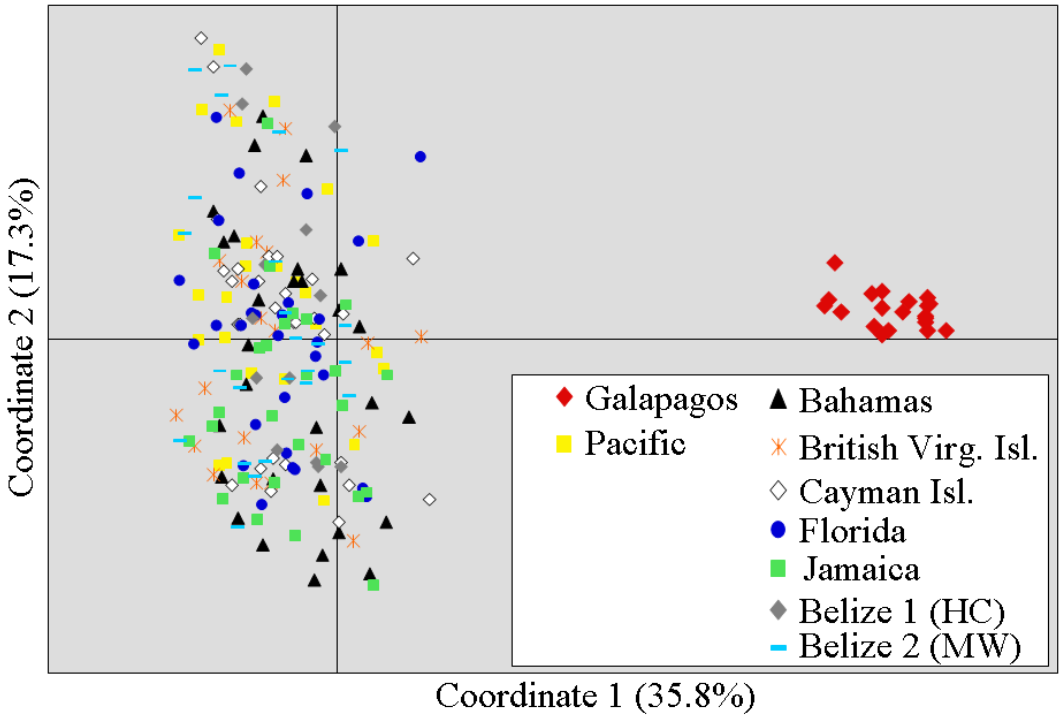
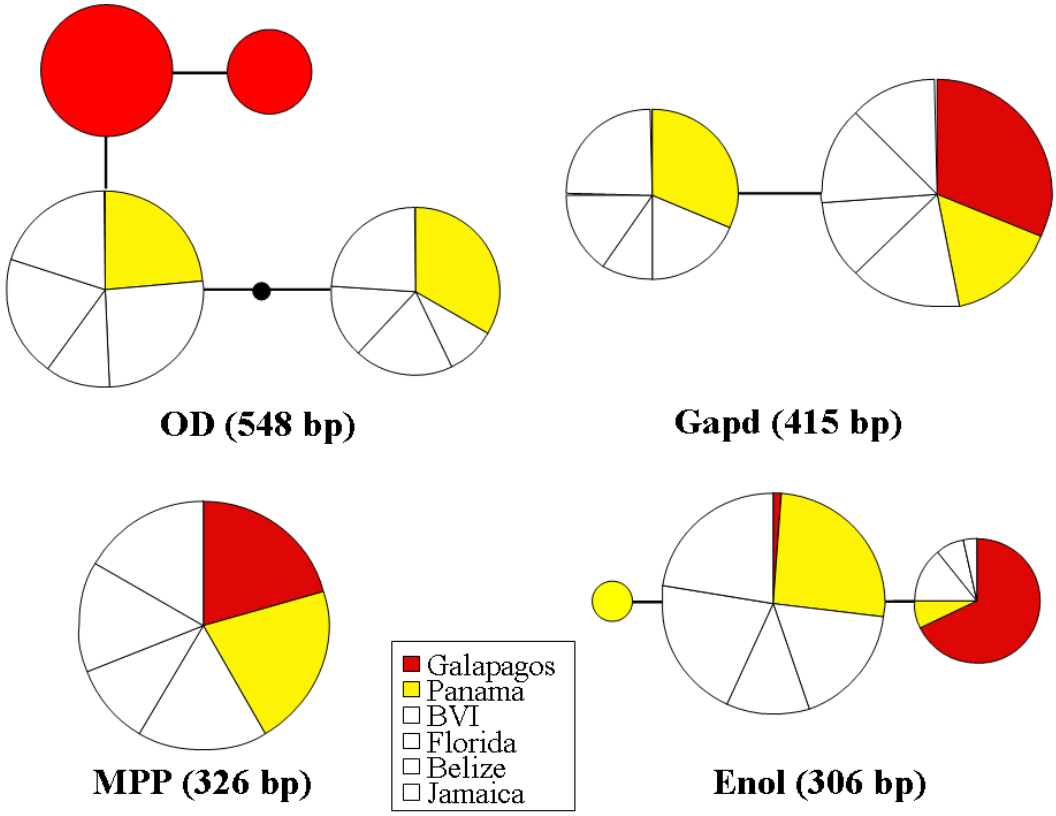

Coordinate 2 (17.3%)
Coordinate 1 (35.8%)
Galapagos
Pacific
Bahamas
British Virg. Isl.
Cayman Isl.
Florida
Jamaica
Belize 1 (HC)
Belize 2 (MW)

Figure 4


OD (548 bp)
Gapd (415 bp)
MPP (326 bp)
Enol (306 bp)
Galapagos
Panama
BVI
Florida
Belize
Jamaica

Online supplementary information for Hailer et al.: Long-term isolation of a highly mobile seabird on the Galapagos. *Proceedings of the Royal Society, Series B.*

Details of Methods

Mitochondrial (mt) DNA PCR conditions:

Polymerase chain reactions (PCRs) of modern samples were performed in 15 µL volumes containing 1x PCR buffer II (Applied Biosystems), 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 467 nM of each primer, 0.06 µL of AmpliTaq Gold polymerase (Applied Biosystems) and approximately 10-50 ng of genomic DNA. PCR cycling conditions were 7 min at 95°, followed by 38 cycles of 40 sec at 95°, 40 sec annealing at 60° (for *ATP6*), 52° (*Cyt B*) or 58° (*ND2*), extension at 72° for 45 sec, and a final elongation at 72° for 15 min (Table S2 provides further details and primer sequences).

DNA from museum specimens was amplified using 10 primer pairs targeting shorter fragments, based on primers designed from sequences obtained from modern samples (see Table S2). PCR conditions were adapted to "ancient" conditions, including the use of BSA, a larger reaction volume (25 µL), and higher primer and polymerase concentrations (see Fleischer et al. 2000).

PCR amplification of microsatellite loci:

We initially assessed multiple loci for amplification and variability in Magnificent Frigatebirds, including all markers from Dearborn et al. (2008), three from Duffie et al. (2008), five from de Ponte Machado et al. (2009), and four from Hickman et al. (2008). Annealing temperatures tested were 50 and 56 degrees, other

details of the PCR conditions are given in table S3. PCR products from up to 7 individuals from several populations were run on 2% agarose gels, and successful amplifications were evaluated on a ABI 3130xl sequencer.

PCR amplification of intron loci

All introns were amplified using existing primers (Friesen et al. 1997, 1999), except for OD, for which new primers were designed using PRIMER3 (Rozen & Skaletsky 1999; table S2). PCRs were performed in 15 μL volumes containing 10-50 ng of genomic DNA, 1x PCR buffer II (Applied Biosystems), 0.2 mM of each dNTP, 2.5 mM $MgCl_2$, 467 nM of each primer and 0.06 μL AmpliTaq DNA polymerase. All thermocyler profiles began with 95°C for 7 min followed by thirty eight cycles of 30 s at locus-specific annealing temperature (OD 55 °C, Enol 63 °C, MPP and GAPD 62 °C), 72°C for 50 s, 95°C for 30s, and a final step at the primer specific annealing temp for 1 min and 72°C for 15 min.

Results: Basic variability of the genetic markers

Complete sequences for the three mitochondrial gene fragments (1636 bp) were obtained for 231 of 232 individuals. Amplifications using different primer sets produced identical sequences, no premature stop codons were detected, and the transition-transversion ratio was high. Double peaks in sequences were rarely observed (in seven individuals, in each case at one fragment only), as expected for haploid loci. Further, the main phylogenetic signal remained identical when individual gene fragments (incl. the mtDNA *ND3* gene, which was sequenced for a subset of individuals; not shown) were analyzed. These observations argue against a potential nuclear origin of the sequences (*Numts*; Sorenson & Fleischer 1996). Heteroplasmy has been documented in other seabirds in the order Pelecaniformes

(Steeves et al. 2005); (Morris-Pocock et al. 2010), likely explaining the rare occurrence of double peaks. Fifty of the 1636 sites were variable, resulting in 36 haplotypes (Table S7). Nucleotide and haplotype diversity were 0.00256±0.00025 (S.D.) and 0.817±0.021 (S.D.).

Data for the eight microsatellite loci were gathered for 219 contemporary individuals. Museum toe-pads were only analysed for mtDNA, and two fresh samples did not amplify consistently for the microsatellites. Across the eight loci, we observed 100 alleles (average: 12.5±7.8 S.D.); observed heterozygosity was 64.6%. Tests within geographically defined populations suggested no significant deviations from Hardy-Weinberg or linkage equilibrium ($p>0.05$, following sequential Bonferroni correction; Table 2), so all loci were used in the following analyses.

For the nuclear introns, we obtained 1595 bp of sequence data (*MPP*: 326 bp; *GAPD*: 415 bp; *ENOL*: 306 bp; *OD*: 548 bp) from 96 chromosomes (48 individuals) of Magnificent Frigatebirds: 20 each from the Galapagos and the Pacific Panama populations, and 56 from the Atlantic (see Table S4 for details). *MPP* showed no variation and we recovered only 7 variable sites across the remaining introns, a result consistent with lower substitution rates in nuclear introns compared to mtDNA. Sequences from the OD intron contained a 1-bp indel, that was fixed on the Galapagos, but polymorphic in the non-Galapagos individuals.

Demographic analyses:

Methods:

Calculations of summary statistics (F_S, Fu 1997; F* and D*, Fu & Li 1993), were performed in DnaSP v5 (Librado & Rozas 2009) and ARLEQUIN 3.5.1.2 (Excoffier & Lischer 2010). Significance was assessed by 10,000 replicate coalescent

simulations in DnaSP. We estimated population growth rate using the coalescent-based Bayesian approach in LAMARC 2.1.3 (Kuhner 2006), based on three parallel chains in an adaptive heating scheme.

Results:

Populations from all three geographic regions (Atlantic, Eastern Pacific and Galapagos) showed signatures of an excess of rare mutations in mtDNA sequences (Table S12); pooling the first two regions did not alter the main conclusions. Since F* and D* values differed non-significantly from zero, and F_S values were significantly negative, this indicates a demographic expansion (Ramos-Onsins & Rozas 2002). Results from LAMARC confirmed this interpretation, yielding positive values for the growth rate, and excluding zero in the 95% posterior credibility intervals.

At the nuclear introns, we detected no significant signal of population expansion for single loci, as indicated by values of Fu's F_S (Table S13). However, evidence of population growth was found when all four introns were analyzed jointly in a Bayesian coalescent-based framework in LAMARC. The 95% posterior credibility intervals of the growth parameter spanned 1601-9403 (for pooled Atlantic populations), 855-9170 (pooled Eastern Pacific samples) and 482-9237 (Galapagos). Those ranges were independent of the priors and excluded zero, indicating an overall signal of population growth in all three geographic regions. The wide confidence intervals of those estimates likely reflect a relatively weak genetic signal of population growth at the nuclear intron loci, consistent with the slower mutation rate and lower number of haplotypes at the introns compared to mtDNA.

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Table S1: Catalog data for museum specimens from which mtDNA was amplified using toe-pad samples.

Specimen ID*	Country	Region	Locality	Collection date
CAS 63241	Mexico	Baja California Sur	Arena Point De La Ventana	8/8 1961
CAS 72851	Mexico	Sinaloa	Mazatlan	Jan. 1895
CAS 72852	Mexico	Sinaloa	Mazatlan	Jan. 1895
CAS 72853	Mexico	Sinaloa	Mazatlan	Jan. 1895
CAS 83651	USA	California	Santa Cruz	3/9 1986
USNM 442821	Colombia	Choco	Nuqui, Pacific Coast	1/29 1951
USNM 58808 (illegible)	Mexico	Sinaloa	Mazatlan	18##
USNM 400105	Panama	Los Santos	Monagre, 5 Miles Northeast	3/16 1948
USNM 376002	Panama	Bay of Panama	Archipiélago De Las Perlas	3/4 1944
USNM 376003	Panama	Bay of Panama	Archipiélago De Las Perlas	4/8 1944
USNM 454994	Panama	Bay of Panama	Canal Zone, Farfan Beach	10/5 1953

* CAS – California Academy of Sciences, San Francisco, CA, USA; USNM – National Museum of Natural History, Washington, DC, USA.

Table S2: Primers used in PCR amplifications of mtDNA and nuclear introns.

Primer name	with primer	amplicon length (bp)	primer sequence (5'-3')	reference
*****MaFr_ATP68-2F	-4R		AACCGCACCTTGAACCTGACC	this study
MaFr_ATP68-4R	-2F	237 bp	GGATTAGGGCTCATTTGTGG	this study
MaFr_ATP68-4F	-5R		TCACAAAACAACTAATAATTCCAC	this study
MaFr_ATP68-5R	-4F	233 bp	TGGTAGGAGATGTCCGAGAG	this study
MaFr_ATP68-5F	-2R		CTACGAAACCAACCCACAAC	this study
*****MaFr_ATP68-2R	-5F	200 bp	TGGGGAGTAGGGCGATTGTACC	this study
*****CytBwow	-R1		ATGGGTGGAATGGAATTTTGTC	(1)
MaFrCytB_R1	CytBwow	192 bp	TCGGACAAACCCTAGTTGAATG	this study
MaFr_CytB_F1	-R2		TCTACTGAGAAGCCTCCTCAG	this study
MaFr_CytB_R2	-F1	220 bp	TCGGACGAGGACTCTACTATGG	this study
MaFr_CytB_F2	CytB1anc		CAGGTTTCTTTGTAGAGGTAG	this study
*****CytB1-anc	-F2	256 bp	CCAACATCTCTGCTTGATGAA	(1)
*****MetL	-H1		AAGCTATCGGGCCCATACCCG	(2)
MaFr_ND2_H1	MetL	226 bp	TATTTAACTGCTGCTTCAATGG	this study
MaFr_ND2_L1	-H2		CTCATCTCAAAACCTCATCACC	this study
MaFr_ND2_H2	-L1	143 bp	CTTAGTTGRGTAATGTCTCAC	this study
MaFr_ND2_L2	-H3		TCCAATGCTTGAGCYACAGGAC	this study
MaFr_ND2_H3	-L2	183 bp	GAATTTTATTRCTGTTGATAG	this study
MaFr_ND2_L3	H5766		AGGCTCATCCTTAACTACTGC	this study
*****H5766	-L3	186 bp	GATGAGAAGGCTAGGATTTTKCG	(3)
*****MPP-F			TACATCTACTTTAACACCTGGACCACCTG	(4)
*****MPP-R			TTGCAGATGGAGAGCAGGTTGGAGCC	(4)

*****Gapd-F	ACCTTTAATGCGGGTGCTGGCATTGC	(5)
*****Gapd-R	CATCAAGTCCACAACACGGTTGCTGTA	(5)
*****MaFr_OD-F	GCCATCATCGGAGTTAGGTG	this study
*****MaFr_OD-R	AAGCCAAGTTCAGCCTAAAATG	this study
*****Enol-F	TGGACTTCAAATCCCCCGATGATCCCAGC	(5)
*****Enol-R	CCAGGCACCCCAGTCTACCTGGTCAAA	(5)

* primers used for contemporary blood samples, targeting larger amplicons. Excluding primer sequences, we obtained 531, 550 and 555 bp of *ATP6*, *CytB* and *ND2*, respectively). All other primers were used for PCR amplification of DNA from museum specimen toe-pad samples.

(1) (Fleischer et al., 2006); (2) O. Haddrath 2004, unpublished; (3) (Sorenson et al., 1999); (4) (Friesen et al., 1999); (5) (Friesen et al., 1997)

Table S3: Multiplex PCR conditions for the eight microsatellite loci amplified in Magnificent Frigatebirds.

Multiplex	Locus [a] (clone name)	annealing temperature (°C)	# PCR cycles [b]	amount of each primer (µL)
A	18D11 (Fmin12)	58	38	0.18
	11F01 (Fmin15)			0.15
	27E09 (Fmin17)			0.50
B	06A09 (Fmin14)	58	38	0.20
	13D06 (Fmin16)			0.08
	01D11 (Fmin02)			0.28
C	16C06 (Fmin11)	57	38	0.15
	27F11 (Fmin18)			0.12

[a] (Dearborn et al., 2008).

[b] Amplifications were performed in 10 µL reactions with 2.5 mM $MgCl_2$, 0.5 µL BSA (New England Biolabs), 0.2 mM of each dNTP and 0.08 µL of AmpliTaq Gold polymerase in 1x buffer II (Applied Biosystems).

Table S4: Phased haplotype data from four nuclear intron loci in Magnificent Frigatebirds. Number of chromosomes sequenced (n) and frequencies of each haplotype (Hn) per region and population. See Fig. 4 regarding phylogenetic relationships among haplotypes.

Region/	**n**	**MPP (326 bp)**	**Gapd (415 bp)**		**Enol (306 bp)**			**OD (548 bp)**			
population		**H_1**	**H_1**	**H_2**	**H_1**	**H_2**	**H_3**	**H_1**	**H_2**	**H_3**	**H_4**
Galapagos	**20**	**20**	**0**	**20**	**1**	**19**	**-**	**15**	**5**	**-**	**-**
North Seymour	*20*	*20*	*0*	*20*	*1*	*19*	*-*	*15*	*5*	*-*	*-*
Eastern Pacific	**20**	**20**	**10**	**10**	**17**	**2**	**1**	**-**	**-**	**13**	**7**
Panama	*20*	*20*	*10*	*10*	*17*	*2*	*1*	*-*	*-*	*13*	*7*
Atlantic	**56**	**56**	**22**	**34**	**49**	**7**	**-**	**-**	**-**	**42**	**14**
Brit. Virgin Islands	*16*	*16*	*6*	*10*	*12*	*4*	*-*	*-*	*-*	*14*	*2*
Florida	*10*	*10*	*3*	*7*	*8*	*2*	*-*	*-*	*-*	*6*	*4*
Belize (HC)	*14*	*14*	*5*	*9*	*14*	*0*	*-*	*-*	*-*	*11*	*3*
Jamaica	*16*	*16*	*8*	*8*	*15*	*1*	*-*	*-*	*-*	*11*	*5*
Total	**96**	**96**	**32**	**64**	**67**	**28**	**1**	**15**	**5**	**55**	**21**

Table S5: Settings used for data analysis in MIGRATE 3.0.7. For the employed uniform priors, lower and upper bounds are given.

Marker set	model details [1]	uniform Theta prior	uniform xNm prior	increment (skipped steps)	burnin	MCMC chain length [2]	bounded-adaptive heating [3]
mtDNA	Ts/Tv=47	0 - 0.06	0 - 4,000	200	200,000	2*180,000	4 chains (1-50)
microsatellites [4]	SMM	0-12	0 - 50,000	100	60,000	1*20,000	4 chains (1-50)
nuclear introns [5]	Ts/Tv from jModeltest	0 – 0.03	0 – 5,000	300	500,000	2*300,000	4 chains (1-50)

[1] Ts/Tv – transition/transversion ratio; SMM – stepwise mutation model.

[2] This is given in the form: number of replicate chains * number of recorded steps.

[3] Numbers indicate the total number of chains in the heating scheme, and their respective range of temperatures.

[4] Due to non-stepwise allele sizes at one locus (*Fmin 18*), these analyses utilized seven out of the in total eight microsatellites.

[5] Since one of the sequenced introns (MPP) did not display any variation in Magnificent Frigatebirds, this locus was omitted from the MIGRATE runs.

See published online supplement for Tables S6, S7, S8 (too large for this format)

Table S9: Pairwise mtDNA differentiation among Magnificent Frigatebird populations. Below the diagonal are pairwise Φ_{ST} values based on the K2P distance, corresponding p values (significance assessed by 10100 permutations in ARLEQUIN) are above the diagonal. Significant Φ_{ST} values ($p<0.05$, following sequential Bonferroni correction) are marked by an asterisk. Note that the Galapagos are significantly differentiated from all other populations, and that only three of the remaining comparisons (among non-Galapagos populations) are significant.

Region	**Atlantic**							**Eastern Pacific**		**Galapagos**
Population	**Bahamas**	**British Virgin Islands**	**Cayman Islands**	**Florida**	**Belize 1 (HC)**	**Jamaica**	**Belize 2 (MW)**	**Panama (Pacific)**	**toe-pads (Pacific)**	**North Seymour**
Bahamas	--	0.212	0.114	0.230	0.004	0.002	0.287	0.007	0.006	<0.001
Brit. V.I.	0.018	--	0.647	0.293	0.190	0.132	0.298	0.252	0.147	<0.001
Cayman I.	0.029	-0.015	--	0.531	0.158	0.020	0.217	0.099	0.101	<0.001
Florida	0.013	0.006	-0.007	--	0.046	0.002	0.387	0.008	0.006	<0.001
Belize (HC)	0.206*	0.032	0.035	0.078	--	0.181	0.014	0.122	0.141	<0.001
Jamaica	0.163*	0.027	0.066	0.129*	0.026	--	0.006	0.295	0.353	<0.001
Belize (MW)	0.005	0.007	0.013	<0.001	0.125	0.126	--	0.027	0.011	<0.001
Panama	0.127	0.011	0.032	0.097	0.043	0.005	0.085	--	0.724	<0.001
toe-pads	0.189	0.038	0.051	0.141	0.049	0.001	0.140	-0.030	--	<0.001
Galapagos	0.947*	0.922*	0.911*	0.930*	0.946*	0.901*	0.943*	0.920*	0.924*	--

Table S10: Differentiation at microsatellite loci among Magnificent Frigatebird populations. Below the diagonal are pairwise F_{ST} values (Weir & Cockerham, 1984); corresponding p values (significance as assessed by 1000 permutations) are above the diagonal. Significant F_{ST} values ($p<0.05$, following sequential Bonferroni correction) are marked by an asterisk. Note that the Galapagos are significantly differentiated from all other populations, and that only five of the remaining comparisons (among non-Galapagos populations) are significant.

Region	**Atlantic**							**E. Pacific**	**Galapagos**
Population	**Bahamas**	**British Virgin Islands**	**Cayman Islands**	**Florida**	**Belize 1 (HC)**	**Jamaica**	**Belize 2 (MW)**	**Panama (Pacific)**	**North Seymour**
Bahamas	--	0.078	0.137	0.562	0.177	0.023	0.013	<0.001	<0.001
Brit. V.I.	0.009	--	0.070	0.710	0.404	0.020	0.006	0.013	<0.001
Cayman I.	0.005	0.009	--	0.427	0.501	<0.001	0.101	0.017	<0.001
Florida	-0.001	-0.003	0.000	--	0.365	0.038	0.030	0.002	<0.001
Belize (HC)	0.008	0.002	-0.001	0.001	--	0.010	0.126	0.162	<0.001
Jamaica	0.011	0.014	0.020*	0.010	0.025	--		<0.001	<0.001
Belize (MW)	0.018	0.026	0.008	0.012	0.012	0.042*	--	0.023	<0.001
Panama	0.030*	0.017	0.013	0.023*	0.008	0.043*	0.015	--	<0.001
Galapagos	0.343*	0.362*	0.351*	0.348*	0.356*	0.358*	0.375*	0.371*	--

Table S11: Assignment test of magnificent frigatebirds in GENECLASS based on microsatellite markers. Numbers denote the count of individuals sampled in the populations in rows, assigned to the populations in columns.

	Galap-agos	Panama	Bahamas	Florida	British Virgin Isl.	Jamaica	Cayman Isl.	Belize (HC)	Belize (MW)
Galapagos	20	-	-	-	-	-	-	-	-
Panama	-	6	2	-	1	1	5	6	4
Bahamas	-	3	9	6	-	6	2	3	-
Florida	-	2	4	4	7	1	3	5	3
Brit.V.Isl.	-	3	3	5	8	2	-	1	-
Jamaica	-	2	1	5	6	3	6	2	3
Cayman Isl.	-	3	3	4	3	3	9	4	1
Belize (HC)	-	3	1	2	2	-	5	-	-
Belize (MW)	-	4	4	1	1	-	4	1	9

Table S12: Demographic analyses of mtDNA data in Magnificent Frigatebirds. N and N_H denote the number of individuals sequenced, and the number of encountered haplotypes, followed by Fu's FS, Fu and Li's F* and D*, and the growth parameter estimated in LAMARC (95% posterior credibility intervals).

Population	n	N_H	F_S	F*	D*	Growth
Galapagos	20	3	-1.863*	-2.18846 $^{n.s.}$	-2.05308 $^{n.s.}$	(13 – 9995)
Eastern Pacific	36	11	-2.876 $^{n.s.}$	-1.96494 $^{n.s.}$	-1.99457 $^{n.s.}$	(460 – 9073)
Atlantic	175	26	-15.738***	-1.85987 $^{n.s.}$	-1.55799 $^{n.s.}$	(2272 – 13475)

* $p<0.05$, ** $p<0.01$, *** $p<0.001$, $^{n.s.}$ non-significant

Table S13: Genetic diversity at four nuclear introns in Magnificent Frigatebirds. Significance ($p<0.05$) is indicated by an asterisk next to the corresponding values, or by n.s. (non-significant).

	MPP	Gapd	Enol	OD
π (nucleotide diversity±S.D.) ($\cdot 10^5$)	0	108±8	143±14	173±17
Galapagos	0	0	33±29	72±18
non-Galapagos	0	119±5	78±20	74±9
Fu's F_S	-	2.049 n.s.	0.447 n.s.	1.258 n.s.
Galapagos	-	-	-0.879 n.s.	0.976 n.s.
non-Galapagos	-	2.149	-0.864 n.s.	1.707 n.s.
Fu & Li's D*	-	0.495 n.s.	-1.061 n.s.	0.830 n.s.
Galapagos	-	-	-1.540 n.s.	0.650 n.s
non-Galapagos	-	0.510 n.s.	-1.004 n.s.	0.511 n.s.
Fu & Li's F*	-	0.931 n.s.	-0.789 n.s.	1.080 n.s.
Galapagos	-	-	-1.648 n.s.	0.765 n.s.
non-Galapagos	-	1.011 n.s.	-1.055 n.s.	0.829 n.s.

Figure S1: Bayesian clustering results using the 'Locprior' model in STRUCTURE 2.3.2. Individual genotypes are shown as vertical columns, with membership to K genetic clusters depicted in different colours.

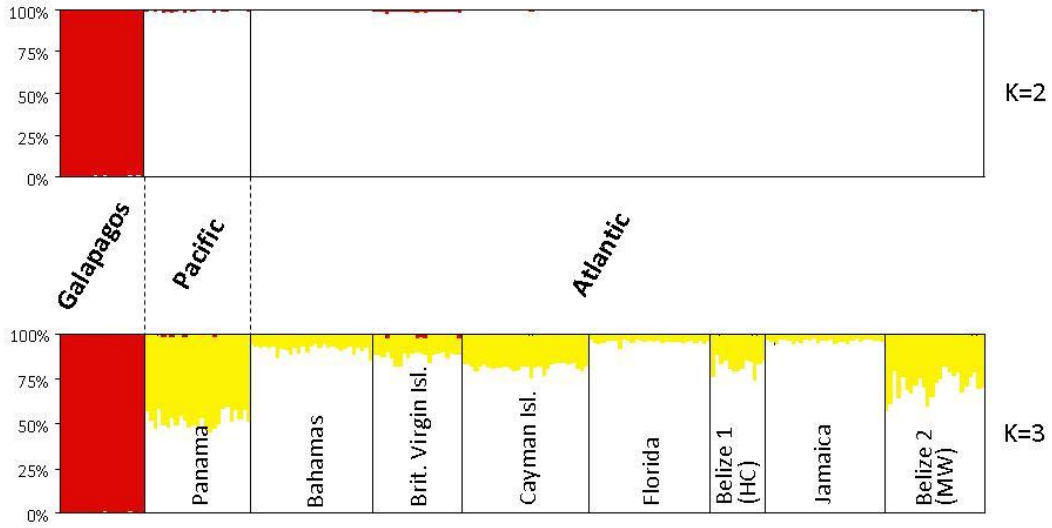

Figure S2: Measurement of middle toe on museum skins. The more commonly assessed ‘tarsus length’ was not possible for us to measure on museum specimens, since the feet of most individuals were not stretched out. The arrows mark the beginning and end points of the middle toe measurement, spanning the two most distal phalangeal bones. Measurement started at the beginning of skin cover on the most distal bone (claw base), and ended proximally at the joint between the second and first bone. Note that all museums skins we measured had (almost) completely extended middle toes, so the measurement on a straight line should have yielded little error from the actual length.

Chapter V: Hippoboscid-transmitted *Haemoproteus* parasites (Haemosporida) infect Galapagos Pelecaniform birds: Evidence from Molecular and morphological studies, with description of *Haemoproteus iwa*

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Abstract: Haemosporidian parasites are widely distributed and common parasites of birds, and the application of molecular techniques has revealed remarkable diversity among their lineages. Four haemosporidian genera infect avian hosts (*Plasmodium*, *Haemoproteus*, *Leucocytozoon* and *Fallisia*), and *Haemoproteus* is split into two sub-genera based on morphological evidence and phylogenetic support for two divergent sister clades. One clade (*Haemoproteus* (*Parahaemoproteus*)) contains parasites developing in birds belonging to several different orders, except pigeons and doves (Columbiformes), while the other (*Haemoproteus* (*Haemoproteus*)) has previously been shown to only infect dove hosts. Here we provide molecular and morphological identification of *Haemoproteus* parasites from several seabird species that are closely related to those found in dove hosts. We also document a deeply divergent clade with two haemosporidian lineages recovered primarily from frigatebirds (Fregatidae, Pelecaniformes) that is sister to the hippoboscid- (Hippoboscidae) transmitted dove parasites. One of the lineages in this new clade of parasites belongs to *Haemoproteus iwa* and is distributed in two species of frigatebird (*Fregata*) hosts from Hawaii, the Galapagos Islands, the eastern Pacific and throughout the Caribbean Basin. Haemosporidian parasites are often considered rare in seabirds due in part to the lack

or low activity of some dipteran vectors (e.g., mosquitos, biting midges) in marine and coastal environments; however, we show that *H. iwa* is prevalent and is very likely vectored among frigatebirds by hippoboscid flies which are abundant on frigatebirds and other seabirds. This study supports the existence of two sister clades of avian *Haemoproteus* in accord with the subgeneric classification of avian hemoproteids. Description of *H. iwa* from Galapagos *Fregata minor* is given based on morphology of blood stages and segments of the mitochondrial cytochrome *b* gene, which can be used for identification. This study shows that hippoboscid flies warrant more attention as vectors of avian *Haemoproteus* spp., particularly in marine and coastal environments.

✯Note: Nucleotide sequence data reported in this paper are available in the GenBank™ database under accession numbers **JF833042** – **JF833066**

1. Introduction

Haemosporidian parasites are ecologically successful apicomplexans (protists) found in birds, reptiles and mammals from nearly all regions of the world aside from those close to the poles (Valkiūnas, 2005). Parasitologists have described numerous genera and subgenera within the order Haemosporida (phylum: Apicomplexa) containing several hundred named species and at least 500 mtDNA haplotypes (Bensch et al., 2009). These parasites are vector-borne and have been associated with transmission by species from at least seven families of Diptera (Levine, 1988). Avian haemosporidians include parasites from four genera: *Plasmodium*, which is typically vectored by mosquitoes (Culicidae); *Haemoproteus*, which is primarily transmitted by biting midges (Ceratopogonidae) and louse flies (Hippoboscidae); *Leucocytozoon*,

which is vectored primarily by blackflies (Simuliidae)(only *L. caulleryi* is known to be transmitted by biting midges); and *Fallisia*, whose vectors are still unclear (Valkiūnas 2005). The application of molecular techniques to the study of haemosporidian parasites has revealed a remarkable amount of genetic diversity, suggesting the existence of many undescribed (in many cases probably cryptic) species that share convergent morphological traits with described taxa (Ricklefs and Fallon, 2002; Bensch et al., 2004; Križanauskienė et al., 2006).

A recent reconstruction of the phylogeny of haemosporidian parasites using sequence data from four genes from each of the parasites' three genomes (nuclear, mitochondrial, plastid) and spanning lizard, bird and mammal parasites (Martinsen et al., 2008) suggests two non-sister clades within avian *Haemoproteus*. One clade (represented by three sequences of *Haemoproteus columbae* in Martinsen et al., 2008) consists of parasites belonging to *Haemoproteus* (*Haemoproteus*) found in doves and is sister to all other ingroup taxa while other avian haemoproteids, (*Haemoproteus* (*Parahaemoproteus*)) found in non-columbiform hosts, form a clade that is sister to *Plasmodium* in mammals, birds and lizards (Martinsen et al., 2008). Santiago-Alarcon et al. (2010) documented additional diversity in *Haemoproteus* (subgenus *Haemoproteus*).

Haemosporidian parasites are common in continental regions but some species also occur on islands. Island populations of potential hosts are often more susceptible to introduced pathogens, as they have historically been exposed to fewer pathogens than mainland populations (e.g., Fromont et al., 2001). The Galapagos Islands are located on the equator approximately 1000 km west of continental

Ecuador and have only been inhabited by humans for 200 years. Much of their biodiversity remains intact, with only 5% species loss (Gibbs et al., 1999). The isolation and high degree of endemism in the biota raise concerns about the introduction of diseases. Introduced pathogens, including avian pox (*Avipoxvirus*) and avian malaria (*Plasmodium relictum*) are a likely cause of major population declines and extinctions (Smith et al., 2006) (see effects of *P. relictum* on the Hawaiian avifauna; van Riper et al., 1986, 2002; Atkinson et al., 2000). Ongoing disease monitoring is an essential part of conservation efforts in Galapagos (Parker et al., 2006). A health survey of four Galapagos seabirds was conducted on the island of Genovesa in 2004 to establish species-specific baseline health parameters for future recognition of health-related threats to the endemic populations (Padilla et al., 2006). The survey discovered *Haemoproteus* sp. blood parasites infecting three of the four seabird species sampled (Great Frigatebird *Fregata minor*, Red-footed Booby *Sula sula* and Swallow-tailed Gull *Creagrus furcatus*). Parasite prevalence, estimated through microscopic examination of blood smears, ranged from 9% to 29% in the different bird species (Padilla et al., 2006). Blood parasites are considered rare in seabirds (e.g., Jovani et al., 2001), which might be related to competent immune defenses made possible by their long embryonic development periods (Ricklefs, 1992) or the lower abundance and/or low activity of some dipteran vectors (e.g., mosquitos, biting midges) in marine environments due to windy conditions and high salinity (Piersma, 1997; Mendes et al., 2005). Only a handful of published studies document *Haemoproteus* spp. in seabirds, three of which report *Haemoproteus* parasites in frigatebirds: Great Frigatebirds in Hawaii (Work and Rameyer, 1996),

Christmas Island Frigatebirds (*Fregata andrewsi*) (Quillfeldt et al., 2010) and Magnificent Frigatebirds (*Fregata magnificens*) in Mexico (Madsen et al., 2007a). In Galapagos, haemosporidian parasites have previously been identified in the Galapagos Dove (*Zenaida galapagoensis*), which has high prevalence and intensity infections and is known to move readily throughout the archipelago (Padilla et al., 2004; Santiago-Alarcon et al., 2006, 2008). Recently, a *Plasmodium* sp. parasite has been identified in Galapagos Penguins (*Spheniscus mendiculus*), which could potentially have negative consequences for the small and vulnerable penguin population (Levin et al., 2009).

Here we present a phylogeny of the blood parasites found in Galapagos birds, which reveals a new clade of *Haemoproteus* parasites found primarily in frigatebirds. The lineage in Galapagos frigatebirds was identified as *Haemoproteus iwa*. Because the original description of this parasite from Hawaiian birds (Work and Rameyer, 1996) is incomplete (there is no information about microgametocytes and only one macrogametocyte was illustrated), we provide a morphological description of blood stages of *H. iwa* from its type avian host *F. minor* in Galapagos. These samples are the same lineage as recorded in Hawaii (the type locality of *H. iwa*). In addition, we provide molecular evidence potentially identifying the vector of *H. iwa*. Using sequences that include those from known morphospecies of described haemosporidian parasites (e.g., Valkiūnas et al., 2007, 2008a, 2010), we are able to understand the placement of this new parasite clade relative to other known lineages (including other Galapagos lineages).

2. Materials and methods

2.1. Sample collection

Samples from Galapagos birds were collected between 2001 and 2010 on numerous field expeditions. Seabirds were captured by hand on the nest or near nesting sites. A blood sample was collected from the brachial vein and stored in lysis buffer. Hippoboscid flies were collected directly from birds while sampling. Flies were stored in 95% ethanol in the field and later at 4°C in the laboratory until DNA extraction. Blood films collected in 2010 were air-dried within 5-10 s after their preparation. They were fixed in absolute methanol in the field and then stained with Giemsa in the laboratory. Blood samples of Magnificent Frigatebirds from Pacific Panama, Belize and the Cayman Islands were collected during the nesting seasons of 2007 and 2008. All samples were from chicks or adults tending active nests. Blood samples from Hawaiian Great Frigatebirds (both breeding adults and juveniles) were collected during the breeding season of 1999 from birds nesting or roosting on Tern Island.

2.2. Molecular screening

DNA was extracted from blood using a standard phenol-chloroform extraction protocol (Sambrook et al., 1989) and PCR was used to amplify regions of the parasite mitochondrial *cytochrome b* gene (mtDNA, *cyt b*). Positive and negative controls were always used and, in most cases, any individual sample that amplified was reamplified to confirm a true positive. Primers used to amplify and sequence parasite *cyt b* from birds tested in the University of Missouri – St. Louis, USA laboratory included an initial outer reaction (HAEMNF and HAEMNR2) followed by an internal

re-amplification (HAEMF and HAEMR2) (Waldenström et al., 2004). Reaction conditions for both sets of primers followed Waldenström et al. (2004). PCRs were performed using Takara Ex taq polymerase and accompanying reagents (Takara Bio Inc, Japan). One microliter of stock DNA was used in the initial reaction and 0.5 μL of amplicon from the initial reaction was used as a template for the internal re-amplification reaction. PCR products were cleaned using Qiagen PCR Purification kits (QIAGEN) or using Exonuclease I and Antarctic Phosphatase (#M0289S and #M0293S, respectively, New England Bio Labs, Inc.). Four hundred and ninety-eight bp of double-stranded DNA sequence were obtained using an Applied Biosystems 3100 DNA Analyzer at the University of Missouri – St. Louis with BigDye Terminator v3.1 Cycle Sequencing chemistry. The protocol used to amplify and sequence parasite DNA from Galapagos seabirds tested at the University of Leeds, UK, also followed Waldenström et al. (2004), but used an annealing temperature of 52˚ C in the internal reaction. For the University of Leeds samples, either Biotaq (Bioline, USA) or Flexi Go Taq (Promega, USA) DNA polymerase was used in these reactions. Samples were sequenced using an Applied Biosystems 3730 DNA Analyzer at the Medical School at the University of Sheffield, UK, with BigDye Terminator v3.1 Cycle Sequencing chemistry. Sequences were obtained from haemosporidian parasites from 4 *Fregata minor* (eight from Hawaii and 56 from Galapagos), 18 *Fregata magnificens* (10 from Galapagos, two from Pacific Panama, two from Belize, four from the Cayman Islands), two *Spheniscus mendiculus* (Galapagos), seven *Zenaida galapagoensis* (Galapagos), five *Sula nebouxii* (Galapagos), two *Creagrus furcatus* (Galapagos) and five *Olfersia* spp. hippoboscid

flies (Galapagos). Twenty-nine additional frigatebird parasites (26 *F. minor* and three *F. magnificens*) were also sequenced using the *caseinolytic protease* gene (*ClpC*) following Martinsen et al. (2008).

In the laboratory, thoraces of 20 hippoboscid flies were carefully separated from heads and abdomens. Each thorax was used individually for DNA extraction using a Qiagen DNEasy Blood and Tissue DNA extraction kit (Qiagen, USA). The standard protocol was followed, but DNA was eluted in half as much buffer due to assumed low concentrations of any parasite DNA. Protocols for PCR amplification and sequencing were as described above. To ensure that the positive PCR results from insects were DNA from sporozoites and not from undigested parasite-infected blood cells that might have persisted in the vector digestive system as remnants of a blood meal, thoraces of the seven insects that tested positive for *Haemoproteus* were tested for the bird mitochondrial *cyt b* gene with primers and protocols used in Ngo and Kramer (2003). Frigatebird mtDNA was used as a positive control to identify and compare bird DNA amplified from insect thoraces. New sequences were deposited in GenBank (accession numbers: **JF833042-JF833066**).

2.3. Phylogenetic analyses

Cyt b sequences were edited in Seqman 4.0 [DNASTAR, USA], added to a larger dataset containing additional *cyt b* sequence data obtained from GenBank (Supplementary Table S1), and aligned using BioEdit (Version 7.0.9.0; Hall, 1999). The best-fit model of DNA evolution was determined using jMODELTEST (Version 0.1.1) (Guindon and Gascuel, 2003; Posada, 2008). The GTR+I+ Γ model of nucleotide substitution was used to reconstruct a maximum likelihood phylogeny and

a maximum likelihood bootstrap analysis (500 pseudoreplicates) (Jobb, 2009; Treefinder http://www.treefinder.de). Bayesian posterior probabilities were obtained from 10 million trees using the program BEAST (Drummond and Rambaut, 2007). BEAST initiates a pre-burn-in to stabilize likelihood values, after which it begins sampling. The likelihood stationarity of sampled trees was determined graphically using TRACER. Parameters in BEAST allow for mutation rate heterogeneity among branches of the phylogeny, reducing bias due to disproportionately long branches (relaxed clock: uncorrelated lognormal). Lineage birth was modeled using a Yule prior. Sequence divergence between the different lineages was calculated in MEGA 3.1 (Kumar et al., 2004).

2.4. Microscopic examination

Blood films were examined for 10-15 min at low magnification (×400) and then at least 100 fields were studied at high magnification (×1,000). Detailed protocols of preparation, fixation, staining and microscopic examination of blood films are described by Valkiūnas (2008b). Intensity of infection was estimated as a percentage by counting the number of parasites per 1,000 red blood cells or per 10,000 red blood cells if infections were light, i.e., <0.1%, as recommended by Godfrey et al. (1987). To determine the possible presence of simultaneous infections with other haemosporidian parasites in the type voucher material of *H. iwa*, the entire blood films were examined microscopically at low magnification.

An Olympus BX61 light microscope (Olympus, Tokyo, Japan) equipped with an Olympus DP70 digital camera and imaging software AnalySIS FIVE (Olympus Soft Imaging Solution GmbH, Münster, Germany) was used to examine slides,

prepare illustrations and to take measurements. The morphometric features studied (Table 1) are those defined by Valkiūnas (2005). Morphology of *H. iwa* from Galapagos material was compared with the parahapantotypes of *H. iwa* (Accession Nos. **G212808**, **G212809** and **G212810** in the Queensland Museum, Brisbane, Queensland, Australia). The student's *t*-test for independent samples was used to determine statistical significance between mean linear measurements. A *P*-value of 0.05 or less was considered significant.

3. Results

3.1. Phylogenetic analyses

Our phylogenetic analyses suggest two major groups, *Plasmodium* and *Haemoproteus*, with *Haemoproteus* further split into two divergent sister clades *Haemoproteus* (*Haemoproteus*) and *Haemoproteus* (*Parahaemoproteus*) (Fig. 1). Clade A contains parasites found primarily in pigeons and doves (*Haemoproteus* (*Haemoproteus*)), which is sister to a new clade of parasites found primarily in frigatebird hosts (clade B) (Fig. 1). Lineages of haemosporidian parasites from both *Plasmodium* and *Haemoproteus* were found in Galapagos birds. Five sequences from Blue-footed Boobies (*S. nebouxii*) clustered with *Haemoproteus* (*Parahaemoproteus*) and *Plasmodium* parasites were found in Galapagos penguins (*S. mendiculus*). The Blue-footed Booby parasite sequences were generated using the same primers as those used to amplify other Galapagos seabird parasites, with no indication of mixed infections (e.g., no double peaks in the chromatogram). Most of the recorded sequences cluster with *Haemoproteus* (*Haemoproteus*) and are split between two major clades (labeled A and B). Clade A contains parasites from Rock Pigeons

(*Columba livia* infected with *Haemoproteus columbae*) (non-Galapagos sequences that have been used in other studies to represent the *H.* (*Haemoproteus*) sub-genus), Galapagos Doves (numerous lineages of *Haemoproteus multipigmentatus*) and unidentified *Haemoproteus* lineages from three seabird species (Nazca Booby (*Sula granti*), Magnificent Frigatebird and Swallow-tailed Gull, (*C. furcatus*) (clade A). Hippoboscid flies and frigatebirds (*F. minor* and *F. magnificens*) from Galapagos, Hawaii (*F. minor*), Caribbean (Belize and Cayman Islands) (*F. magnificens*) and Pacific coasts of Panama (*F. magnificens*) as well as one Swallow-Tailed Gull (*C. furcatus*, also from Galapagos) were infected with *Haemoproteus* parasites that formed a well-supported and hitherto undescribed clade (clade B) which is sister to clade A (Fig. 1). Average pairwise sequence divergence between clade A and clade B is 8%. There is no genetic variation among all sequences from frigatebird parasites (clade B); as mentioned above, one Magnificent Frigatebird parasite sequence clustered with the clade A containing mostly dove parasites, while all others (n = 82) were identical for the *cyt b* fragment sequenced and encountered in Pacific and Caribbean *F. minor* (n = 8 from Hawaii and n = 56 from Galapagos) and *F. magnificens* (n = 10, 2, 2, 4 from Galapagos, Pacific Panama, Belize and Cayman Islands, respectively). To avoid redundancy, only one to two from each species/location of these sequences are shown in Fig. 1. Parasites from all Galapagos frigatebirds were morphologically identical; they belong to *H. iwa* (see description below).

We obtained 20 *Haemoproteus ClpC* sequences from Galapagos frigatebirds, seven from Hawaiian frigatebirds and two from Caribbean/Pacific Panamanian

frigatebirds and found that the results were consistent with the *cyt b* gene; there was no variation in clade B containing primarily frigatebird parasites, which form a well supported clade as with *cyt b*.

Seven parasite DNA sequences were recovered from thoraces of hippoboscid flies collected from Great Frigatebirds and they were identical to the lineage found in clade B (Fig. 1). It is unlikely that the detected parasite DNA was from gametocytes remaining in blood meals because no bird DNA could be amplified from the thoraces.

3.2. Description of Haemoproteus (Haemoproteus) iwa Work and Rameyer, 1996 from Fregata minor in the Galapagos Islands

Young gametocytes: Earliest forms were not seen in voucher material.

Macrogametocytes (Fig. 2A- 2H)*:* Extend along nuclei of erythrocytes and displace the nuclei laterally from early stages of their development (Fig. 2A-2C), which is a characteristic feature of parasite development. Elongate broadly-halteridial bodies with even or slightly irregular outline, but more frequently the former; ameboid forms not seen. Cytoplasm blue, homogeneous in appearance, often possesses prominent vacuoles of variable size (Figs. 2B-2E, 2H); volutin granules not seen. Both growing (Figs. 2A, 2B) and fully-grown gametocytes (Figs. 2E, 2F) appressed to erythrocyte envelope but do not touch erythrocyte nuclei. A few fully-grown gametocytes were seen in association with erythrocyte nuclei; if present, such association is superficial and often disconnected at 1 or several points (Figs. 2G, 2H). Parasite nucleus markedly variable in form, frequently irregular in shape, submedial or medial in position (Figs. 2A-2H). Nucleolus frequently seen (Fig. 2C); occasionally, 2 nucleolus-like clumps of chromatin were visible (Fig. H). Pigment

granules of small (< 0.5 μm) and medium (0.5-1 μm) size, roundish, irregular or oval in form, black, very numerous (Table 1), randomly scattered throughout cytoplasm. Size and number of pigment granules increase as parasite matures (compare Figs. 2A-2C and 2E-2H). Fully-grown gametocytes only slightly enclose erythrocyte nuclei with their ends, filling erythrocytes up to their poles (Figs. 2E-2H); they markedly displace nuclei of erythrocytes laterally (Figs. 2F, 2G), frequently to envelope of erythrocytes (Fig. 2H). Infected erythrocytes are hypertrophied and their nuclei atrophied in length, width and area compared with uninfected erythrocytes (Table 1, $P < 0.01$ for all of these characters).

Microgametocytes (Figs. 2I-2L): General configuration as for macrogametocytes with usual hemosporidian sexually dimorphic characters. Gametocytes do not touch erythrocyte nuclei; this feature is more evident in fully-grown microgametocytes than in macrogametocytes (compare Figs. 2F-2H and 2J-2L). Outline more irregular and fewer vacuoles than in macrogametocytes (compare Figs. 2A-2H and 2I-2L); ameboid forms present (Fig. 2L). Cytoplasm is of reddish shade, partly due to markedly diffuse parasite nuclei, boundaries of which are unclear, making nuclei difficult to measure. Number of pigment granules is approximately one-half that in macrogametocytes (Table 1, $P < 0.001$). Pigment granules lighter in color (usually brown) than in macrogametocytes; the majority of granules tend to group and to gather close to ends of gametocytes, but individual granules can be seen anywhere in the cytoplasm (Figs. 2K, 2L). Fully-grown microgametocytes are more slender in form and displace host nuclei less than macrogametocytes (Table 1, compare Figs. 2G, 2H and 2K, 2L).

3.2.1. Taxonomic summary

Avian hosts: *Fregata minor*, *F. magnificens* (Pelecaniformes).

Distribution: H. iwa and its *cyt b* lineages were recorded on Hawaii, Galapagos, Eastern Pacific and Caribbean coast/islands; it is probably widespread in the range of distribution of frigatebirds.

Voucher specimens: Blood films (intensity of parasitemia is approximately 0.01%, *Fregata minor*, North Seymour, Galapagos, 00°23'38" S, 90°17'32" W, lineage FminGal1, collected by I. Levin, 6 July 2010) are deposited in the Institute of Ecology, Nature Research Centre, Vilnius, Lithuania (Accession Nos. 47740 NS, 47741 NS), in the U. S. National Parasite Collection, Beltsville, Maryland, USA (USNPC 104268, 104269), and in the Queensland Museum, Brisbane, Australia (G465451, G465452).

Additional material: Thirty-two slides (Accession Nos. 47744 – 47775 NS) where intensity of parasitemia is < 0.001%, other data as for voucher specimens, are deposited in the Institute of Ecology, Nature Research Centre, Vilnius, Lithuania. Duplicates of these slides are also available at the University of Missouri – St. Louis, USA.

DNA sequences: Mitochondrial *cyt b* lineage FminGal1 (GenBank™ Accession No. **JF833050**) can be used for molecular identification of *H. iwa.*

Vector: Olfersia spinifera (Diptera, Hippoboscidae) is a probable vector in Galapagos.

Prevalence: In Galapagos, the overall prevalence of infection in Great Frigatebirds based on PCR detection was 113 of 204 (55.4%).

3.2.2. Remarks

Haemoproteus iwa can be readily distinguished from other avian hemoproteids due to the large number of pigment granules in its macrogametocytes (Table 1), which is approached only by *H. multipigmentatus* in the columbiform, *Z. galapagoensis* (see Valkiūnas et al., 2010); the former species nonetheless produces more pigment granules (average number of the granules in macrogametocytes of these parasites is 57 and 43, respectively, $P < 0.001$). Interestingly, both of these parasites produce more pigment granules in macrogametocytes than any other described species of avian hemoproteid, and are therefore similar from this point of view. In *H. iwa* i) mature gametocytes are often not appressed to erythrocyte nuclei, which is particularly evident in microgametocytes (see Figs. 2J-2L), ii) macrogametocytes often possess prominent vacuoles (Figs. 2A-2E, 2H), and iii) the number of pigment granules in macrogametocytes is at least twice that in microgametocytes (Table 1). None of these readily distinguishable features of *H. iwa* are characteristics of *H. multipigmentatus*.

A full range of blood stages of *H. iwa* (except earliest gametocytes) is published for the first time (Figs. 2A-2L). Microgametocytes were not reported in the original description of *H. iwa*, probably due to extremely light infection (Work and Rameyer, 1996), but they are present in voucher material of this parasite from Galapagos (Figs. 2I-2L). Macrogametocytes are more numerous than microgametocytes; the ratio in the voucher material is 2.5 : 1.

4. Discussion

According to current knowledge, parasites from the subgenus *Haemoproteus* (currently only seven species defined mainly by morphological and life history traits) infect birds only from the order Columbiformes (Valkiūnas et al., 2010). Thus, this is the first time that close phylogenetic relatives of parasites belonging to the subgenus *Haemoproteus* have been found and documented both by PCR and microscopy in non-columbiform hosts. This parasite is *H. iwa*, which is the first representative of the subgenus *Haemoproteus* infecting non-columbiform birds. *Haemoproteus iwa* was originally described from the Great Frigatebird in Hawaii (Work and Rameyer, 1996); the original description is incomplete (microgametocytes were not described) and is based on extremely light infections (only four gametocytes of the parasite were seen in this species' parahapantotype material after a 4 h examination, G. Valkiūnas personal observation). During this study, we detected the same lineages of *H. iwa* in Great Frigatebirds in both Hawaii and Galapagos. Because i) parasitemia was relatively high, ii) the main morphological features of Hawaiian and Galapagos parasites are similar, and iii) the same *cyt b* haplotype was present in Great Frigatebirds in Hawaii and Galapagos, our material provided an opportunity to prepare a morphological re-description of *H. iwa* that is important for future taxonomic and ecological studies.

While some of the seabirds (Nazca Booby NZB9, Magnificent Frigatebird CY18, Swallow-Tailed Gull STG14; see Fig. 1) appear to be infected by parasite lineages very similar in DNA sequence to *H. multipigmentatus* infecting the Galapagos doves (clade A), the majority of the frigatebirds (and one Swallow-Tailed

Gull) are infected with parasites that form their own, well-supported sister clade within the subgenus *Haemoproteus* (clade B). Diversity reported in clade B has never been described, perhaps due to under-representation in sampling for molecular studies of parasites infecting marine and coastal birds. The detection of what is likely *H. multipigmentatus* in the occasional seabird (*S. granti*, *C. furcatus*, clade A) could represent sporozoites injected into the bloodstream from a bite by *Microlynchia galapagoensis*, the Hippoboscid fly normally parasitizing doves (Valkiūnas, 2010). Doves were seen near seabird colonies (I. Levin, personal observation) and PCR protocols can amplify sporozoites from the peripheral blood of birds (Valkiūnas et al., 2009). It remains unclear whether *H. multipigmentatus* can complete development in seabirds to gametocyte stage. Thus, the detection of parasite DNA in the blood does not provide evidence that the parasite can complete its lifecycle in these seabird species. This warrants further investigation and exemplifies the need for studies that include both molecular and microscopical approaches.

Clade B does not appear to be unique to the Galapagos, as DNA sequences from parasites infecting Hawaiian, Pacific Panamanian and Caribbean Magnificent Frigatebirds have the same sequence as parasites in Galapagos frigatebirds. Thus, *H. iwa* has a wide range of distribution and infects different species of frigatebirds. This is similar to the results found for *H. multipigmentatus* infecting Columbiformes, where this parasite is not endemic to the Galapagos but is widely distributed across the American continent (Santiago-Alarcon et al., 2010). Based on molecular evidence (Fig. 1, clade B), it is possible that *H. iwa* also completes development in the gull, *C. furcatus*, but detection of blood stages is needed for confirmation.

In order to assess the lack of sequence diversity in *cyt b* (one parasite haplotype for all clade B frigatebird parasites), we amplified and sequenced a portion of the parasite's plastid genome, *ClpC*, for a subset of samples. Santiago-Alarcon et al. (2010) found that *ClpC* was more variable at the tips of the parasite phylogeny; thus, it provided a better resolution of the relationships among haplotypes of *H.* (*Haemoproteus*) spp. in doves when *cyt b* did not (see also Outlaw and Ricklefs, 2010). We obtained 29 *Haemoproteus ClpC* sequences from Galapagos, Hawaiian, Panamanian and Caribbean frigatebirds and found that the results were consistent with the *cyt b* gene; we observed no variation in clade B sequences containing primarily parasites of frigatebirds. In contrast, Great Frigatebirds from Hawaii and Galapagos show strong genetic differentiation at mitochondrial and nuclear loci (Hailer et al., unpublished data). Furthermore, within Magnificent Frigatebirds, the Galapagos population has apparently been isolated from conspecific populations in the Pacific and Atlantic since the Pleistocene era (Hailer et al., 2010). In the light of these findings, sharing of the same *H. iwa* lineage among frigatebirds from diverse geographic locations reported here suggests either a very slow rate of sequence evolution in clade B, or transmission of the parasite among frigatebird populations in the absence of host gene flow.

A possible sequence divergence rate for haemosporidian *cyt b* has recently been estimated at 1.2% per million years for lineages infecting passerine birds (Ricklefs and Outlaw, 2010). Using this estimate (assuming the rate also applies to haemosporidians of non-passeriform birds) and colonization times of Magnificent Frigatebirds to the Galapagos calculated by Hailer et al. (2010), we can estimate the

probability that Galapagos lineages would not have diverged since the host colonized the archipelago. Based on a geometric mean colonization time of 247,200 years before the present, the probability of no nucleotide changes in 524 bp of *cyt b* since colonization is 0.21. For the 95% confidence limits of the frigatebird colonization time (Hailer et al., 2010), we estimated the probability of no divergence to be 0.59 for the most recent colonization estimate (82,800 years before present (YBP)) and 0.015 for the most ancient (647,400 YBP). Therefore, the absence of differentiation between this and source lineages of the frigatebird haemosporidian is not incompatible with arrival of the parasite with the colonizing population of frigatebirds.

The well-supported clade of primarily frigatebird haemosporidian parasites, which is sister to clades of hippoboscid-transmitted *H. columbae* and *H. multipigmentatus*, indicates that subgeneric classification of haemoproteids remains valid and we cannot continue to consider *H.* (*Haemoproteus*) blood parasites to be columbiform-specific. Importantly, *H. iwa* haplotypes were present in thoraces of hippoboscid flies. A possibility for transmission of this parasite by hippoboscid flies was speculated by Work and Rameyer (1996) and Valkiūnas (2005, p. 861), but there has been no supporting evidence for this to date. Although several recent publications have reported blood parasites in non-passerines (e.g., Mendes et al., 2005; Krone et al., 2008; Ishak et al., 2008, Ortego et al., 2008; Outlaw and Ricklefs, 2009; Yohannas et al., 2009; Quillfeldt et al., 2010), none of these have identified parasites belonging to, or closely related to, the subgenus *Haemoproteus*.

Haemoproteids of the subgenera *Haemoproteus* and *Parahaemoproteus* are transmitted by different groups of vectors and undergo markedly different sporogony, and therefore differ genetically and appear in different clades in phylogenetic trees (Martinsen et al., 2008; Santiago-Alarcon et al., 2010). Briefly, species of *H.* (*Haemoproteus*) are transmitted by flies belonging to the Hippoboscidae and are characterized by large oocysts (>20 μm in diameter) that possess numerous germinal centers, many sporozoites in mature oocysts (>500) and relatively short sporozoites (mean less than 10 μm) that are usually blunt at one end and pointed at the other (Baker, 1966; Garnham, 1966; Atkinson, 1991; Valkiūnas, 2005). None of these characteristics are features of *H.* (*Parahaemoproteus*) spp. Development of *H. iwa* in hippoboscid flies remains undescribed.

Concerning *H. iwa*, a possibility for transmission by hippoboscid flies was speculated by Work and Rameyer (1996) and Valkiūnas (2005, p. 861). Here we provide molecular evidence that suggests that hippoboscid flies (*Olfersia* sp., probably *Olfersia spinifera* from reports of this fly parasitizing frigatebirds) are the vectors for *H. iwa* among frigatebirds, based on identical parasite DNA sequences amplified from hippoboscid thoraces. These ectoparasitic flies are common on frigatebirds and related pelecaniforms, even in the dry climates of Galapagos coastal habitat (I. Levin, personal observation). Because parasite DNA, but no bird DNA, was recovered from fly thoraces, it is likely that the sequences came from the sporozoites of *H. iwa.* The sporozoite is the only sporogonic stage in avian haemosporidians that is present in thoraces and salivary glands of the vectors, including hippoboscid flies (Baker, 1966; Valkiūnas, 2005). Biting midges have also

been documented as vectors for *Haemoproteus* parasites; however, they have not been caught in traps near seabird colonies in Galapagos (J. Rabenold, personal communication) Biting midges typically require higher humidity and are therefore less likely to occur at these dry and windy coastal sites. Our molecular evidence and ecological observations provide strong support for *Olfersia* sp. hippoboscid flies as the vector for *H. iwa*, but detection of oocysts in the mid-gut and sporozoites in the salivary glands of the flies ideally followed by experimental infection of uninfected seabirds by sporozoites would be necessary for complete confirmation of the vector.

Given that Galapagos frigatebird *H. iwa* parasites were identical at this region of *cyt b* to parasites from frigatebirds across the New World tropics – despite the genetic isolation of the Galapagos Magnificent Frigatebird – it is possible that the parasite is being moved between populations of frigatebirds during the non-breeding season via the transfer of the hippoboscid fly vectors at roosting sites where populations of frigatebirds might interact but apparently do not interbreed. Given this possibility, we confirmed the infection status of chicks and juvenile *F. minor* from Galapagos. Five of 20 chicks and 18 of 22 juveniles were infected with *H. iwa*, providing evidence that this lineage is locally transmitted in the Galapagos.

It is unclear whether hemoproteids pose a health threat to their Galapagos hosts. *Haemoproteus* parasites are typically considered benign by most veterinarians, but recent experimental evidence shows some fitness consequences for infected hosts in the wild (e.g., Merino et al., 2000; Marzal et al., 2005). It is important to note that some species of avian *Haemoproteus* cause severe pathology in birds (Cardona et al., 2001) and are sometimes lethal (Ferrell et al., 2007). Additionally, male Magnificent

Frigatebirds infected with *H. iwa* tended to have lighter colored red gular pouches (Madsen et al., 2007a), although there is not evidence for a role of gular pouch color in mate choice in frigatebirds (Madsen et al., 2007b). Finally, Galapagos Great Frigatebirds infected with *Haemoproteus* spp. had significantly higher heterophil-to-lymphocyte concentration ratios than uninfected individuals, indicating that they were physiologically stressed or actively fighting an infection (Padilla et al., 2006). Further studies are needed to understand the pathogenicity of *H. iwa*.

In conclusion, we have documented *H. iwa* and closely related lineages of haemosporidian parasites from Galapagos seabirds that are closely related to parasites that have previously only been found in dove and pigeon hosts. In addition, we have provided molecular evidence for a deeply divergent haemosporidian clade recovered primarily from frigatebirds that is sister to the dove and pigeon parasite clade. These parasites from frigatebirds show no genetic variation at *cyt b*, even across broad geographic scales. We provide evidence that *H. iwa* is likely vectored by the hippoboscid fly, *O. spinifera*, which is abundant on frigatebirds and other seabirds (Work and Rameyer, 1996; Quillfeldt et al., 2010). Characterizing these parasites by placing them in a phylogenetic context with other previously described taxa is the first step in understanding their evolutionary history and their host breadth. Importantly, molecular evidence from this study shows that species of the Hippoboscidae are likely vectors not only of *H. iwa* but also of avian *Haemoproteus* spp. of other marine and coastal birds (Fig. 1 clades A and B). This finding indicates that hippoboscid flies warrant more attention as possible vectors of hemoproteids among not only columbiform birds, but also among non-columbiform birds,

particularly in marine and coastal environments. Future studies should focus on the population level transmission dynamics of these haemosporidian parasites and explore the role of the vector in moving the parasites across large geographic distances as these parasite genetic data might suggest. The striking contrast between the genetically isolated Galapagos frigatebird host and the very widespread parasite is interesting and unexpected, and warrants future research.

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Table 1.

Morphometry of host cells and fully-grown gametocytes of *Haemoproteus iwa* from the great frigatebird *Fregata minor*.

Feature	Measurements (μm) [a]
Uninfected erythrocyte	
Length	14.3-16.6 (15.2±0.5)
Width	6.6-8.4 (7.6±0.5)
Area	84.5-108.0 (95.1±6.6)
Uninfected erythrocyte nucleus	
Length	6.1-8.2 (7.0±0.5)
Width	2.1-3.7 (2.6±0.4)
Area	10.7-19.4 (14.5±2.3)
Macrogametocyte	
Infected erythrocyte	
Length	13.2-17.7 (16.2±1.2)
Width	6.9-10.2 (8.3±0.9)
Area	78.8-123.5 (108.6±9.8)
Infected erythrocyte nucleus	
Length	5.7-7.3 (6.8±0.4)
Width	2.1-2.7 (2.3±0.1)
Area	9.7-15.6 (13.5±1.5)

Gametocyte	
Length	15.5-19.6 (17.9±1.1)
Width	3.3-5.7 (4.3±0.6)
Area	60.1-82.0 (74.0±5.2)
Gametocyte nucleus	
Length	2.6-4.4 (3.5±0.5)
Width	1.8-3.4 (2.4±0.4)
Area	3.9-8.0 (6.2±1.2)
Number of pigment granules	49-67 (57.4±5.1)
NDR[b]	0.2-0.5 (0.4±0.1)
Microgametocyte	
Infected erythrocyte	
Length	13.0-18.0 (15.3±1.5)
Width	7.1-11.0 (8.5±0.9)
Area	87.3-133.7 (105.6±12.5)
Infected erythrocyte nucleus	
Length	6.1-8.2 (7.2±0.6)
Width	1.9-2.9 (2.3±0.3)
Area	11.6-16.2 (14.1±1.1)
Gametocyte	
Length	14.6-20.9 (17.3±1.6)

Width	3.0-4.2 (3.5±0.3)
Area	40.5-74.7 (53.2±8.9)
Gametocyte nucleus[c]	
Length	-
Width	-
Area	-
Pigment granules	25-40 (31.7±3.4)
NDR	0.5-0.9 (0.7±0.1)

[a]All measurements (*n* = 21) are given in micrometers. Minimum and maximum values are provided, followed in parentheses by the arithmetic mean and S.D.

[b]NDR = nucleus displacement ration according to Bennett and Campbell (1972).

[c]Due to a markedly diffuse nucleus, its measurement is difficult (see description of the parasite, section 3.2)

Figure Legends

Fig. 1. Maximum likelihood (ML) phylogenetic hypothesis of haemosporidian parasites based on 524 bp of the mitochondrial *cyt b* gene. ML bootstrap values appear above the nodes and Bayesian posterior probabilities appear below the nodes. Clades A and B belong to the subgenus *Haemoproteus*; sequences in clade A are mostly parasite lineages restricted to Columbiformes, whereas clade B sequences are parasite lineages restricted to frigatebirds (with one exception of one lineage found in a Swallow-Tailed Gull). For previously unpublished sequences, host species appear in parentheses; sequences from Galapagos are bolded. Parasite lineages are detailed in Supplementary Table S1 and listed in the order in which they appear in the phylogeny.

Figs. 2. *Haemoproteus* (*Haemoproteus) iwa* from the blood of the Great Frigatebird *Fregata minor* in Galapagos. A-H – macrogametocytes, I-L – microgametocytes. Long arrows – nuclei of parasites, short arrows – unfilled spaces among gametocytes and nuclei of infected erythrocytes. Large arrow head – nucleolus. Small arrow heads – vacuoles. Giemsa-stained thin blood films. Bar = 10 μm.

Figure 1

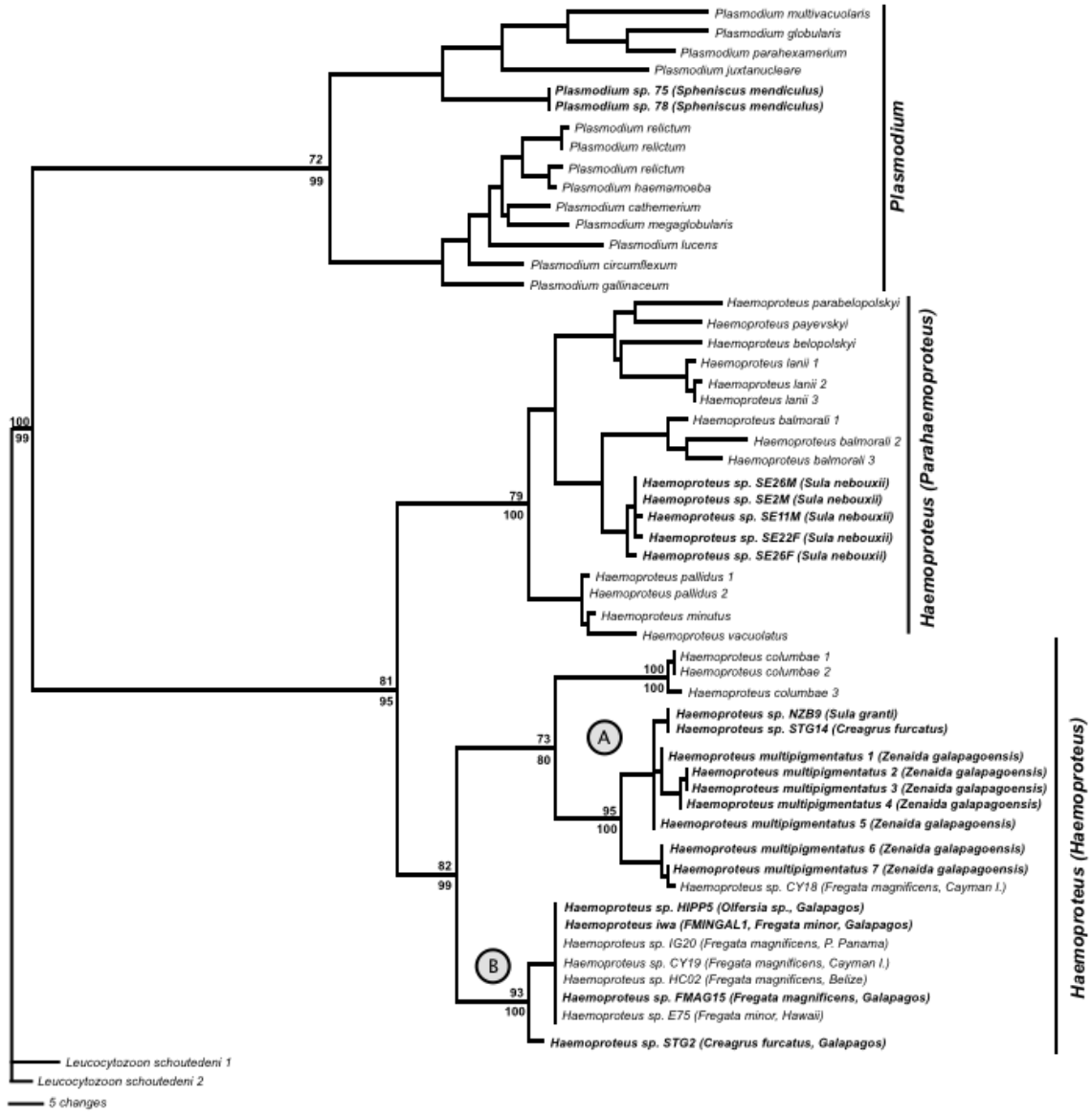
Plasmodium multivacuolaris
Plasmodium globulariis
Plasmodium parahexamerium
Plasmodium juxtanucleare
Plasmodium sp. 75 (Spheniscus mendiculus)
Plasmodium sp. 78 (Spheniscus mendiculus)
Plasmodium relictum
Plasmodium relictum
Plasmodium relictum
Plasmodium haemamoeba
Plasmodium cathemerium
Plasmodium megaglobulariis
Plasmodium lucens
Plasmodium circumflexum
Plasmodium gallinaceum
Plasmodium
Haemoproteus parabelopolskyi
Haemoproteus payevskyi
Haemoproteus belopolskyi
Haemoproteus lanii 1
Haemoproteus lanii 2
Haemoproteus lanii 3
Haemoproteus balmorali 1
Haemoproteus balmorali 2
Haemoproteus balmorali 3
Haemoproteus sp. SE26M (Sula nebouxii)
Haemoproteus sp. SE2M (Sula nebouxii)
Haemoproteus sp. SE11M (Sula nebouxii)
Haemoproteus sp. SE22F (Sula nebouxii)
Haemoproteus sp. SE26F (Sula nebouxii)
Haemoproteus pallidus 1
Haemoproteus pallidus 2
Haemoproteus minutus
Haemoproteus vacuolatus
Haemoproteus (Parahaemoproteus)
Haemoproteus columbae 1
Haemoproteus columbae 2
Haemoproteus columbae 3
Haemoproteus sp. NZB9 (Sula granti)
Haemoproteus sp. STG14 (Creagrus furcatus)
A
Haemoproteus multipigmentatus 1 (Zenaida galapagoensis)
Haemoproteus multipigmentatus 2 (Zenaida galapagoensis)
Haemoproteus multipigmentatus 3 (Zenaida galapagoensis)
Haemoproteus multipigmentatus 4 (Zenaida galapagoensis)
Haemoproteus multipigmentatus 5 (Zenaida galapagoensis)
Haemoproteus multipigmentatus 6 (Zenaida galapagoensis)
Haemoproteus multipigmentatus 7 (Zenaida galapagoensis)
Haemoproteus sp. CY18 (Fregata magnificens, Cayman I.)
Haemoproteus sp. HiPP5 (Olfersia sp., Galapagos)
Haemoproteus iwa (FMINGAL1, Fregata minor, Galapagos)
Haemoproteus sp. IG20 (Fregata magnificens, P. Panama)
Haemoproteus sp. CY19 (Fregata magnificens, Cayman I.)
Haemoproteus sp. HC02 (Fregata magnificens, Belize)
B
Haemoproteus sp. FMAG15 (Fregata magnificens, Galapagos)
Haemoproteus sp. E75 (Fregata minor, Hawaii)
Haemoproteus sp. STG2 (Creagrus furcatus, Galapagos)
Haemoproteus (Haemoproteus)
Leucocytozoon schoutedeni 1
Leucocytozoon schoutedeni 2
5 changes
72
99
100
99
79
100
81
95
100
100
73
80
95
100
82
99
93
100

Figure 2

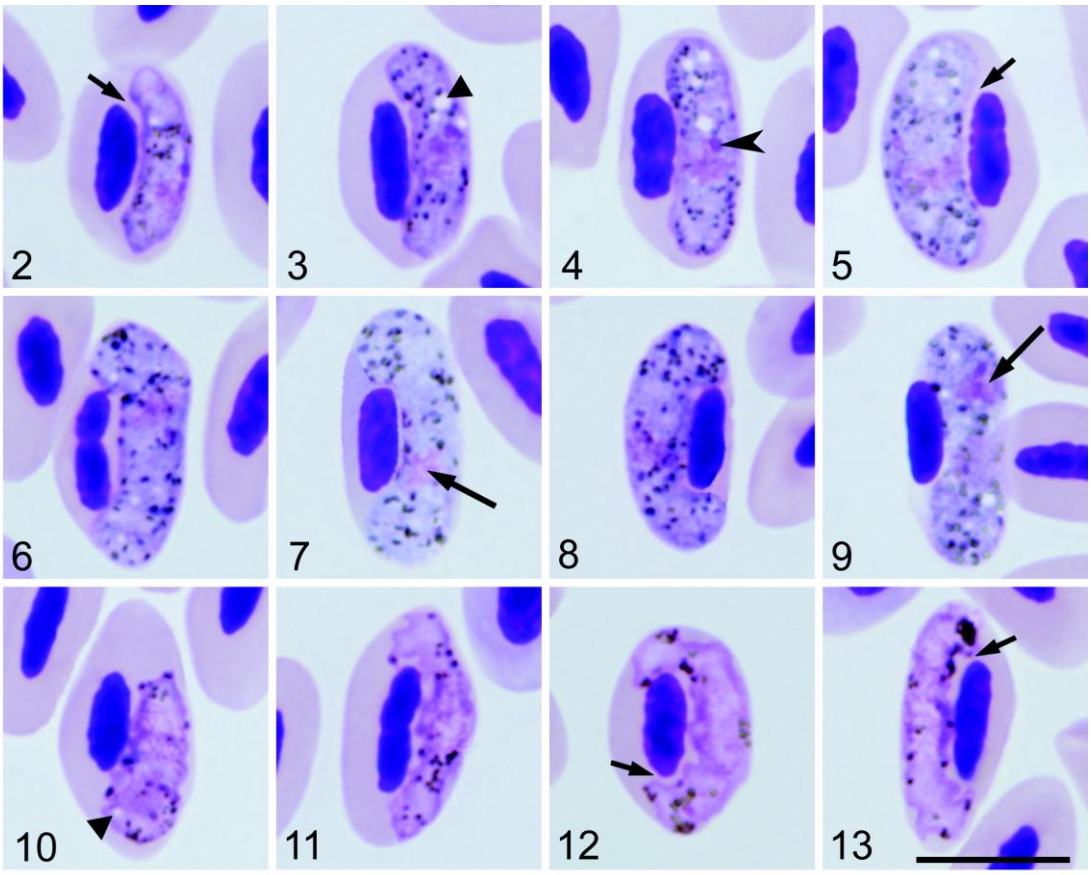

Table S1: DNA sequences used in phylogeny.

SEQUENCE NAME	ASSESSION NUMBER	CITATION
Plasmodium multivacuolaris	**FJ389157**	Valkiūnas et al., 2009
Plasmodium globularis	**EU770151**	Valkiūnas et al., 2008
Plasmodium parahexamerium	**FJ389155**	Valkiūnas et al., 2009
Plasmodium juxtanucleare	**AB302893**	Murata et al., 2008
Plasmodium sp. 75	**JF833046**	Present study
Plasmodium sp. 78	**JF833047**	Present study
Plasmodium relictum	**AF495571**	Waldenström et al., 2002
Plasmodium relictum	**AY831748**	Perez-Tris and Bensch, 2005
Plasmodium relictum	**AY099041**	Perkins and Schall, 2002
Plasmodium haemamoeba	**DQ368378**	Perez-Tris et al., 2007
Plasmodium cathemerium	**AY377128**	Wiersch et al., 2005
Plasmodium megaglobularis	**EU770152**	Valkiūnas et al., 2008
Plasmodium lucens	**FJ389156**	Valkiūnas et al., 2009
Plasmodium circumflexum	**AF495576**	Waldenström et al., 2002
Plasmodium gallinaceum	**AY099029**	Perkins and Schall, 2002
Haemoproteus parabelopolskyi	**AY831751**	Perez-Tris and Bensch, 2005
Haemoproteus payevkyi	**DQ630009**	Hellgren et al., 2007
Haemoproteus belopolskyi	**DQ630006**	Hellgren et al., 2007
Haemoproteus lanii 1	**DQ630010**	Hellgren et al., 2007
Haemoproteus lanii 2	**DQ630011**	Hellgren et al., 2007
Haemoproteus lanii 3	**DQ630012**	Hellgren et al., 2007
Haemoproteus bamorali 1	**DQ630007**	Hellgren et al., 2007
Haemoproteus balmorali 2	**DQ630008**	Hellgren et al., 2007
Haemoproteus balmorali 3	**DQ630014**	Hellgren et al., 2007
Haemoproteus SE26M	**JF833064**	Present study
Haemoproteus sp. SE2M	**JF833060**	Present study
Haemoproteus sp. SE11M	**JF833061**	Present study
Haemoproteus sp. SE22F	**JF833062**	Present study
Haemoproteus sp. SE26F	**JF833063**	Present study
Haemoproteus pallidus 1	**DQ630004**	Hellgren et al., 2007
Haemoproteus pallidus 2	**DQ630005**	Hellgren et al., 2007
Haemoproteus minutus	**DQ630013**	Hellgren et al., 2007
Haemoproteus vacuolatus	**EU770153**	Valkiūnas et al., 2008
Haemoproteus columbae 1	**EU254548**	Martinsen et al., 2008
Haemoproteus columbae 2	**EU254549**	Martinsen et al., 2008
Haemoproteus columbae 3	**EU254553**	Martinsen et al., 2008
Haemoproteus sp. NZB9	**JF833059**	Present study
Haemoproteus sp. STG14	**JF833066**	Present study
Haemoproteus multipigmentatus 1	**JF833051**	Present study
Haemoproteus multipigmentatus 2	**JF833052**	Present study
Haemoproteus multipigmentatus 3	**JF833053**	Present study
Haemoproteus multipigmentatus 4	**JF833054**	Present study

Haemoproteus multipigmentatus 5	**JF833055**	Present study
Haemoproteus multipigmentatus 6	**F833056**	Present study
Haemoproteus multipigmentatus 7	**F833057**	Present study
Haemoproteus sp. CY18	**F833042**	Present study
Haemoproteus sp. HIPP5	**F833049**	Present study
Haemoproteus iwa FMINGAL1	**F833050**	Present study
Haemoproteus sp. IG20	**F833058**	Present study
Haemoproteus sp. CY19	**F833043**	Present study
Haemoproteus sp. HC02	**F833048**	Present study
Haemoproteus sp. FMAG15	**F833045**	Present study
Haemoproteus sp. E75	**F833044**	Present study
Haemoproteus sp. STG2	**F833065**	Present study
Leucocytozoon schoutedeni 1	**DQ67823**	Sehgal et al., 2004
Leucocytozoon schoutedeni 2	**DQ67824**	Sehgal et al,. 2004

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Chapter VI: Prevalence of *Haemoproteus iwa* in Galapagos Great Frigatebirds (*Fregata minor*) and their obligate fly ectoparasite (*Olfersia spinifera*)

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ABSTRACT: Prevalence of haemosporidian parasites varies among different host species, geographic locations, habitats**,** and host life histories, and yet we do not have a firm understanding of the ultimate causes of the variation. Seabirds are not typically found infected with haemosporidian parasites; however, frigatebird species have been repeatedly documented with *Haemoproteus* infections. *Haemoproteus iwa*, in Galapagos great frigatebirds (*Fregata minor*), is vectored by a hippoboscid fly, *Olfersia spinifera*, an obligate ectoparasite of the bird host. Five populations of Galapagos great frigatebirds and flies collected from the birds were sampled and tested for *H. iwa*. Prevalence did not differ across 4 yr or between 5 islands, but males were found to have significantly higher prevalence of infection than females. Additionally, juveniles were more likely to be infected than adults and chicks. Because the invertebrate vector is an obligate parasite, we were able to estimate prevalence in the vector as well as the particular host upon which it fed, a task that is impossible, or nearly impossible, in haemosporidian parasites vectored by midges or mosquitoes. We tested the correlation between the infection status of the bird host and the infection status of the fly collected from the bird. More often than not, the two were correlated, but some mismatches were found. Using the occurrence of infected flies on uninfected birds (12/99) as a proxy for transmission potential, we can estimate the transmission rate to be between 5 and 20% (95% confidence intervals) between individual vertebrate hosts.

Avian haemosporidian parasites are broadly distributed across host taxa and around the world. Some of the most common haemosporidian parasites of birds are species of *Plasmodium*, *Haemoproteus*, and *Leucocytozoon*, which are typically transmitted by mosquitoes (*Plasmodium*), biting midges, and hippoboscid flies (*Haemoproteus*), and black flies (*Leucocytozoon*). Prevalence of infection is reported to vary significantly among bird orders (e.g., Bennett et al., 1993; Valkiūnas, 2005), geographic location (e.g., Bennett et al., 1992; Tella et al., 1999), and habitat (e.g., Figuerola, 1999; Jovani, 2001; Shurulinkov and Chakarov, 2006). However, the ultimate causes of this variation are poorly understood (Scheurerlein and Ricklefs, 2004). There are few generalities, but some recurring patterns include differential prevalence in different age classes of birds (e.g., Sol et al., 2003; Valkiūnas, 2005; van Oers et al., 2010), lower prevalence of haemosporidian parasites in arctic and marine habitats (Bennet et al., 1992), fewer cases of haemosporidian parasites in certain avian orders (raptors (Falconiformes), parrots (Psittaciformes), shorebirds (Charadriiformes), and seabirds (Procellariiformes, Pelecaniformes))(e.g., Peirce and Brooke, 1993; Figuerola et al., 1996, Quillfeldt et al., 2010), as well as some support for a correlation with host life history (Ricklefs, 1992). Slower-developing, longer-lived bird species have been shown in some cases (Ricklefs, 1992; Tella et al., 1999), but not others (Scheuerlein and Ricklefs, 2004), to have fewer haemosporidian parasites, a possible consequence of greater antibody diversity due to a longer incubation period.

Contrary to evidence of low haemosporidian prevalence in marine environments or in long-lived seabirds, there have been 5 published reports of

Haemoproteus in frigatebirds (Pelecaniformes: Fregatidae). Work and Rameyer (1996) described *Haemoproteus iwa* in 35% (21/60) of great frigatebirds (*Fregata minor*) in Hawaii. Padilla et al. (2006) reported 29.2% (7/24) infected *F. minor* on 1 island in the Galapagos. Thirty-nine of 251 (15.5%) magnificent frigatebird (*F. magnificens*) males sampled in Mexico were infected with *H. iwa* (Madsen et al., 2007) and Quillfeldt et al. (2010) found 56% (5/9) prevalence of a *Haemoproteus* species in Christmas Island frigatebirds (*F. andrewsi*). Levin et al. (2011) found *H. iwa* in frigatebirds from the Galapagos Islands, the Hawaiian Islands, the Pacific coast of Panama, and from the Caribbean. Frigatebirds either seem to encounter *Haemoproteus* parasites more frequently, or are more susceptible, than other seabirds, in which haemosporidian infections are uncommon (Merino et al., 1997; Merino and Minguez, 1998; Engström et al., 2000). *Haemoproteus iwa* belongs to the subgenus *Haemoproteus haemoproteus*, which are vectored by hippoboscid flies (Levin et al., 2011), unlike *Haemoproteus parahaemoproteus* species, which are typically vectored by ceratopogonid midges.

To understand the higher prevalences of *Haemoproteus* in frigatebirds, it is important to understand the biology of the parasite both in the vertebrate host, where it is typically detected, and in the arthropod vector, for which we have far less information. The timing of sporogony is different in midge and fly vectors, i.e., sporogony in biting midges is usually complete in less than 10 days (correlated with the gonadotropic cycle of the midge so that the parasite's infective stage is present for the subsequent blood meal), while sporogony in hippoboscid flies is not necessarily synchronized with blood meals and tends to be more prolonged (Valkiūnas, 2005).

This difference is probably associated with both the relatively long life of the fly and the close association of the fly with the vertebrate host (most are obligate bird parasites).

Flies belonging to the Hippoboscidae are highly specialized obligate parasites of birds and mammals. Unlike their well-known relative, the tsetse fly (Glossinidae), Hippoboscidae spend all or nearly all of their adult life on the host. Their dorsoventrally flattened morphology makes them well adapted to live amongst bird feathers and, while most Hippoboscidae species have fully developed and functional wings, they tend to stay closely associated with the host. Hippoboscid species belonging to *Olfersia* are typically found parasitizing frigatebirds (Maa, 1969). *Olfersia spinifera* [Leach 1817] is often called the frigatebird fly, but has been known to parasitize cormorants (Phalacrocoracidae), boobies (Sulidae), pelicans (Pelecanidae), and gulls (Laridae).

This study reports *H. iwa* prevalence in great frigatebirds breeding throughout the Galapagos Archipelago. The Galapagos Islands (Fig. 1) are located approximately 1,000 km off the coast of Ecuador and are nesting habitat for many seabirds, including both great and magnificent frigatebirds (*Fregata magnificens*). Galapagos frigatebirds appear to be genetically isolated from other conspecifics throughout their range. Great frigatebirds from Galapagos and Hawaii show strong genetic differentiation at mitochondrial and nuclear loci (F. Hailer et al., unpubl. obs.) and within magnificent frigatebirds, the Galapagos population has apparently been isolated from conspecific populations in the Pacific and Atlantic since the middle, or late, Pleistocene (Hailer et al., 2011). Galapagos seabirds are infected with several

lineages of haemosporidian parasites, but the most common parasite (and the only one found in Galapagos *F. minor*) is *H. iwa* (Levin et al., 2011). Here, we examine prevalence in *F. minor*, the vertebrate host, with respect to island, yr, sex, and age class (chick, juvenile, adult). Additionally, we are able to compare host prevalence data with *H. iwa* prevalence in the hippoboscid flies captured on the birds. This comparison can help us understand the transmission dynamics of *H. iwa* in this system, and add unique insight into the role of the vector in acquiring, maintaining, and transmitting the parasite.

MATERIALS AND METHODS

Three-hundred and three *Fregata minor* were sampled on 5 different islands (Fig. 2) (Darwin (n=15), Española (n=44), Genovesa (n=171), North Seymour (n=58), and Wolf (n=15)) in June and/or July of 2006, 2007, 2008, and 2010 during the incubation and chick-rearing stages. Seabirds were captured by hand on the nest or near nesting sites. Samples from chicks approximately three weeks or older were included in this study. A blood sample was collected from the brachial vein and stored in lysis buffer. Hippoboscid flies were collected directly from birds while sampling and were stored in 95% ethanol in the field and later at 4 C in the laboratory until DNA extraction. Flattened wing chord and weight measurements were taken for each bird and breeding adults' sex was determined based on obvious sexually dimorphic plumage characteristics. Sex of juveniles and chicks was determined by PCR using the universal primers 2550 and 2718 (Fridolfsson and Ellegren, 1999).

DNA extraction, PCR techniques used to amplify parasite DNA, and sequencing follows Levin et al. (2011). Parasite DNA sequences were confirmed as

H. iwa by comparing to an *H. iwa* specimen that has been previously identified using microscopy and DNA sequencing (Levin et al., 2011).

In the laboratory, thoraxes of 105 hippoboscid flies were separated from heads and abdomens. A Qiagen DNEasy Blood and Tissue DNA extraction kit (Qiagen, Valencia, CA) was used to individually extract the DNA from each fly thorax. The standard protocol was followed, but DNA was eluted in half as much buffer due to assumed low concentrations of any parasite DNA. Protocols for PCR amplification and sequencing were as described in Levin et al., 2011. To ensure that the positive PCR results from insects were DNA from sporozoites and not from undigested parasite-infected blood cells that might have persisted in the vector midgut as remnants of a blood meal, thoraxes of all flies were tested for bird mitochondrial *cyt b* gene with primers and protocols used in Ngo and Kramer (2003). We interpreted the PCR-positive flies as carrying infective sporozoites only when they did not also strongly amplify bird DNA in the thorax extracts. Frigatebird mitochondrial DNA was used as a positive control to identify and compare bird DNA amplified from insect thoraxes. In order to assess repeatability of the hemosporidian screen, we re-tested one-third of the 105 bird-fly pairs for parasites.

Chi square tests or Fisher's Exact tests, performed in R v2.13.1, were used to compare prevalences between bird sexes, island, and age class, as well as to compare parasite status of the fly and its corresponding host. Analyses of variance (ANOVA) (R v2.13.1) were used to compare body condition index (residual of mass [g] regressed against wing length [cm]) and parasite status. The body condition index

was calculated separately for each sex due to females weighing on average significantly more than males of similar sizes (as measured by wing length).

RESULTS

Of the 303 sampled *F. minor* individuals, 131 (43%) were adult female, 124 (40%) were adult male, and 48 (16%) were of juveniles (n=26) or chicks (n=22). Across all 303 samples, 147 individuals (49%) tested positive by PCR for *H. iwa*. The efficiency of the PCR screen was good, with 96.8% repeatability when considering the bird samples and 94.3% in flies. No individuals previously identified as uninfected showed up infected when re-tested. Prevalence by island is summarized in Table I. There was no significant difference in parasite prevalence between islands (Chi Square, P=0.24) or between yr (Chi square, P=0.08). Additionally, when looking only at the 2 islands repeated across yr (Genovesa, North Seymour), there was no difference in *H. iwa* prevalence between the 2 yr (Genovesa: Chi square, P=0.23; North Seymour: Chi square, P=0.45).

There was, however, a significant difference between *H. iwa* prevalences among adult males and females (Chi square, P=0.05) (Fig. 2). Adult males were found to be more infected than expected (69/255 infected, 59/255 expected) and adult females had fewer infections than expected (54/255 infected, 63/255 expected). There was no gender difference in prevalence among young birds (chicks and juveniles combined)(Chi square, P=1.0) or between male and female chicks (Fisher's Exact Test, P=0.57) and male and female juveniles (Fisher's Exact Test, P=0.92).

We also found a significant affect of age on parasite status, i.e., juvenile *F. minor* were more likely to be infected, while chicks had fewer infections than

expected (Chi square, *P*=0.006) (Fig. 2). Eighteen of 26 juveniles were infected (12 expected), while only 5 chicks of 22 (10 expected) tested positive. There was no relationship between parasite status (infected, uninfected) and gender-specific body condition index (ANOVA, *P*=0.51, *F*=0.44). There were very large deviations in body condition index that probably reflect fluctuations in weight during incubation bouts, as many of the individuals sampled were either incubating eggs or attending small chicks. No relationship was found between spatial information (distance from focal individual to nearest nest and number of nests within 10 m) and infection status (distance to nearest nest: ANOVA, *P*=0.43, *F*=.0.628, and nests within10 m: ANOVA, *P*=0.47, *F*=0.53).

We captured at least 1 fly from 105 of 303 birds while sampling (Darwin n=10, Española n=42, Genovesa n=23, N. Seymour n=27, Wolf n=3). Bird *cyt b* amplified in 1 of 41 infected flies and in 5 of 64 uninfected flies and these flies and their bird hosts were removed from the analysis. Of the 99 flies, forty (40.4%) were positive for *H. iwa*, 15% lower prevalence than was found in the corresponding hosts (55/99 infected). We found significant departures from expected when comparing the parasite status of flies with their corresponding hosts (Chi square, *P*=0.03, Fisher's Exact Test, *P*=0.02) (Table II). Infected birds were found to have infected flies more often than expected by a random distribution, and the same result was found for uninfected flies on uninfected birds. There were fewer mismatched situations (uninfected flies on infected birds and infected flies on uninfected birds) than expected. The rarest combination was the occurrence of infected flies from uninfected birds (12/99 cases).

DISCUSSION

One of the most robust results from these data is the higher prevalence of *H. iwa* in juvenile *F. minor*. This has previously been shown as an important pattern in host-parasite assemblages (e.g., Graves et al., 1988; Gregory et al., 1992; Allander and Bennett, 1994; Dawson and Bortolotti, 1999). Three, non-mutually exclusive hypotheses have been proposed to explain this pattern: (1) juveniles with heavy parasite load die before becoming adults; (2) the development of acquired immunity reduces the prevalence and/or intensity of parasites in adults; and (3) adults are less exposed to parasites due to differences in behavior. In the case of Galapagos *F. minor*, we know juvenile mortality is not insignificant; we see dead juvenile birds throughout the breeding colonies (I. Levin, pers. obs.). However, it is not possible in this case to relate the mortality to parasites. Juvenile *F. minor* are fed for an extended period of time by both parents, even after they are capable of flying. Therefore, mortality could also be due to abandonment or death of one, or both, parents. We have very little evidence concerning immunity, particularly comparing adult and juvenile *F. minor*; however, Galapagos *F. minor* infected with *H. iwa* had significantly higher heterophil-to-lymphocyte concentration ratios than uninfected individuals, indicating that infected individuals were physiologically stressed and/or actively fighting the infection (Padilla et al., 2006). It is possible that we find lower prevalence in adults compared to juveniles because of immunity or resistance from prior infection. Additionally, a proportion of adult infections are likely recrudescent. As far as differential exposure to parasites between juveniles and adults, it was already mentioned that juveniles are likely more sedentary than adults because they

are fed by a parent for an extended period of time. The more mobile lifestyle of an adult could contribute to fewer obligate fly ectoparasites and, therefore, lower *H. iwa* exposure. Frigatebirds are frequently seen preening while flying, using their feet to scratch their head and neck (Metz and Schreiber 2002), and this may be a successful way to remove flies. Although older chicks were sampled in order to avoid detection problems due to the delay between inoculation and gametocyte production, the low prevalence of *H. iwa* in chicks could still reflect this time delay or simply the reduced probability of infection based on age.

The significant sex bias in prevalence has a few possible explanations. Androgens, particularly testosterone, can be immunosuppressive (e.g., Peters, 2000), resulting in higher prevalence of haemosporidian parasites in males. Contrary to this logic, a review of 33 studies of haemosporidian parasites in birds showed that *Haemoproteus* infections were significantly more common among breeding females than breeding males (McCurdy et al., 1998). It is important to consider the breeding biology of frigatebirds in this case. These birds are strikingly sexually dimorphic and are the most ornamented seabird. Males have a prominent gular pouch that becomes red during the breeding season (Nelson, 1975). Males perform complex mate attraction rituals in dense breeding aggregations, displaying for females who fly above. During this time, adult males may be more susceptible to host-seeking hippobscid flies than adult females. Operational sex ratios in breeding colonies are usually male-biased (5.5 males per female on Tern Island in Hawaii)(Dearborn et al., 2001). It is possible that with these skewed male-female ratios and resulting intense sexual competition and sexual selection, males might allocate more resources towards

condition or sexual ornaments, rather than the suppression of haemosporidian parasites. Female *F. minor* have been shown to incubate for longer bouts than males, and care for fledglings for 1-2 months longer (Dearborn et al., 2001). However, the greater contribution of females to incubation (roughly 10 more days of the 57 day incubation period) could correspond to their larger body size; if males are losing a larger percentage of overall body mass per incubation shift, it could require a longer foraging trip to recover. Given these data, one might still expect females to be more heavily parasitized than males due to their disproportionate share of reproductive effort. Finally, it is important to consider that *H. iwa* might have little, to no, detrimental effect on *F. minor*, even though some *Haemoproteus* species have been experimentally shown to affect reproductive success (Merino et al., 2000; Marzal et al., 2005). While the sample size for chicks and juveniles is small, we detected no sex differences in infection in these age classes, which provides some evidence for the sex differences in adults to be a result of differential resource allocation by adults and/or some physiological difference that emerges upon or after sexual maturity.

There was no effect of year or island on prevalence of *H. iwa* in Galapagos *F. minor* populations. This could be interpreted as a consistency in vector abundance between sites and year. Because the vector for this *Haemoproteus* species is an obligate ectoparasite of the vertebrate host, one can appreciate that there is a lower reliance on suitable vector habitat and microclimate as there might be with a free-living vector of other haemosporidian species, e.g., mosquitoes vectoring *Plasmodium* spp.). Prevalence was consistent between years for the 2 islands sampled twice, both at least 2 years apart. It is worth noting that, in all cases,

sampling occurred during June and July, which is the cool, dry season in the Galapagos and the peak of the breeding season for great frigatebirds in the Galapagos; however, these frigatebirds on different islands do not nest synchronously across the archipelago.

The comparison of infection status of the flies and the bird hosts is interesting and provides relatively unique data for haemosporidian parasites, as most involve a free-living vector. The disproportionate number of infected birds with infected flies and uninfected birds with uninfected flies support the assertion that the fly is very closely associated with the bird host. We found fewer cases of uninfected flies on infected birds than our statistical test would expect by chance, but there were still 27 of 99 cases. Keeping in mind that we are amplifying *H. iwa* DNA from the thorax of the fly, it is conceivable that the parasite might not be in the sporogony phase that occurs in the thorax. Sporogony is more prolonged in hippoboscid-vectored *Haemproteus* (usually longer than 10 days), and less synchronized than one might find with a midge-vectored parasite. Because the vector is continuously feeding on host blood, one would expect a relatively high chance of detecting parasite sporozoites. While we are interpreting DNA amplified from fly thorax as detection of parasite sporozoites, it is also possible that PCR might be detecting migrating ookinetes or gametocytes, which will glue to the apical part of the intestine (G. Valkiunas pers. comm.). Due to the possibility that we could be amplifying more than one parasite stage from the fly thorax extractions, our interpretation must be considered suggestive. However, since only 1 of the 41 positive flies also tested strongly positive by PCR for presence of bird DNA, this increases confidence in our

interpretation, since gametocytes or even ookinetes would be more temporally associated with the presence of bird blood. Currently, there is no evidence that can better guide our interpretations, as these new approaches to studying micro-transmission dynamics are made possible by molecular techniques and are just now being explored in these ways.

Not all vectors are efficient at acquiring, maintaining, and transmitting parasites. In fact, only 10% of the known vectors for *H. columbae* had sporozoites after being fed mature gametocytes (Valkiūnas, 2005). Vector competence does not have to be very high to successfully transmit the parasite, and this could explain our cases of uninfected flies on infected birds, which contribute to a 15% lower prevalence of *H. iwa* in flies than in birds. Flies could also have a variable amount of resistance to *H. iwa*. We were able to detect bird DNA (*cyt b*) in some of the fly thoraxes, which could indicate that when we detect both parasite DNA and avian DNA in the thorax of a single fly, it is possible that the parasite DNA is from a blood meal. In some cases, there was very faint amplification of bird *cyt b*, which would be a result of bird DNA from blood in the abdomen in the dissected thoraxes. Dissections were performed using a microscope with sterile tools, but it is possible that there was some head and/or abdomen contamination in some samples.

One of the puzzling fly-host infection status combinations is an infected fly on an uninfected host, of which we had 12 in 99 samples. There are a few possible explanations. First, we could (as we could with cases of infected birds and uninfected flies) be underestimating *H. iwa* prevalence by not always detecting it accurately with our PCR test. PCR is known to provide both false negatives and positives (e.g.,

Cosgrove et al., 2006; Valkiūnas et al., 2006) and the degree of under- and over-estimation differs between parasite genera and species (Garamszegi, 2010). Looking at mean microscopy estimates in 1,185 species and 441 species where infection was estimated by PCR, Garamszegi (2010) found no significant difference in *Haemoproteus* prevalence (mean by microscopy: 11.6%, mean by PCR: 16.7%). Therefore, it seems PCR does a good job detecting true *Haemoproteus* infections. When we re-tested one-third of our bird-fly pairs, we found high repeatability (96.8% for birds, 94.3% for flies). Assuming we are not underestimating infections in either the bird or the fly host, the occurrence of infected flies on uninfected birds could be a result of an infected fly moving from a nearby (infected) bird.

We found infected flies on uninfected birds in 12% of the bird-fly pairs, which can be interpreted as the lower bound of an estimate of movement between hosts (and, therefore, parasite transmission). We cannot say for sure with these data whether any particular uninfected fly on an uninfected bird originated on another uninfected bird, or whether any infected fly on an infected bird switched from another infected host. Similarly, uninfected flies on infected hosts could indicate inefficient vector competence as discussed above or fly movement. Therefore, 12% is probably an underestimate of fly movement between hosts, but it could still provide a useful starting point to understanding transmission success of *H. iwa*. While the sample size is very small still (n=17 flies), we were able to amplify 4 *F. minor* microsatellite regions in DNA extracted from flies. We compared *F. minor* genotypes amplified from flies to the genotypes of the hosts they were captured on and found 2 of 5 mismatched genotypes in *H. iwa* infected flies from infected birds and 1 of 5

mismatched genotypes in uninfected flies captured from uninfected birds. One host genotype from an uninfected fly did not match its infected bird host, while another clearly had 2 avian genotypes, 1 that matched the host and 1 that did not. One of the infected flies from uninfected birds had a bird genotype that did not match the host from which it was collected, while the other matched. These are preliminary data at this stage, but we are currently pursuing this approach to understanding fine-scale local transmission dynamics and vector movement in this system.

This study provides yet another documentation of high prevalence of *Haemoproteus* in frigatebirds. Based on high prevalence of *H. iwa* in Galapagos *F. minor* fly ectoparasites, we know that *O. spinifera* is efficient at acquiring, maintaining, and transmitting the parasite, and we can estimate the transmission success to be between 5 and 20% (95% confidence intervals) between individual vertebrate hosts. These data are novel in that we can test the infection status of the invertebrate hosts on particular vertebrate hosts, a task that is impossible or nearly impossible in haemosporidian parasites vectored by midges or mosquitoes. The next logical step in this system is to use other direct (mark recapture of hippoboscid flies) or indirect (population genetics studies) approaches to refine our understanding of fly movement and parasite transmission.

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Table I: Prevalence of *Haemoproteus iwa* in Galapagos Great Frigatebirds (*Fregata minor*) sampled from five islands

Island	**Year**	**Prevalence**		
		Infected	*Uninfected*	*n*
Darwin	2008	7	8	15
Española	2007	23	21	44
Genovesa	2006	58	84	142
Genovesa	2008	16	13	29
N. Seymour	2007	20	10	30
N. Seymour	2010	15	13	28
Wolf	2008	8	7	15

Table II: Counts of Great Frigatebird-Hippoboscid fly pairs showing infection status for vector and host.

	BIRD STATUS	
FLY STATUS	*Uninfected*	*Infected*
Uninfected	32	27
Infected	12	28

FIGURE LEGENDS

FIGURE 1. Map of the Galapagos Islands, Ecuador. Islands where sampling was done are labeled.

FIGURE 2. Prevalence of *Haemoproteus iwa* in adult males and adult females (left) and in chicks, juveniles, and adults (right). Prevalence was calculated as the number of infected individuals in a category/total number of individuals in the category. Sample sizes are shown above the bars.

Figure 1

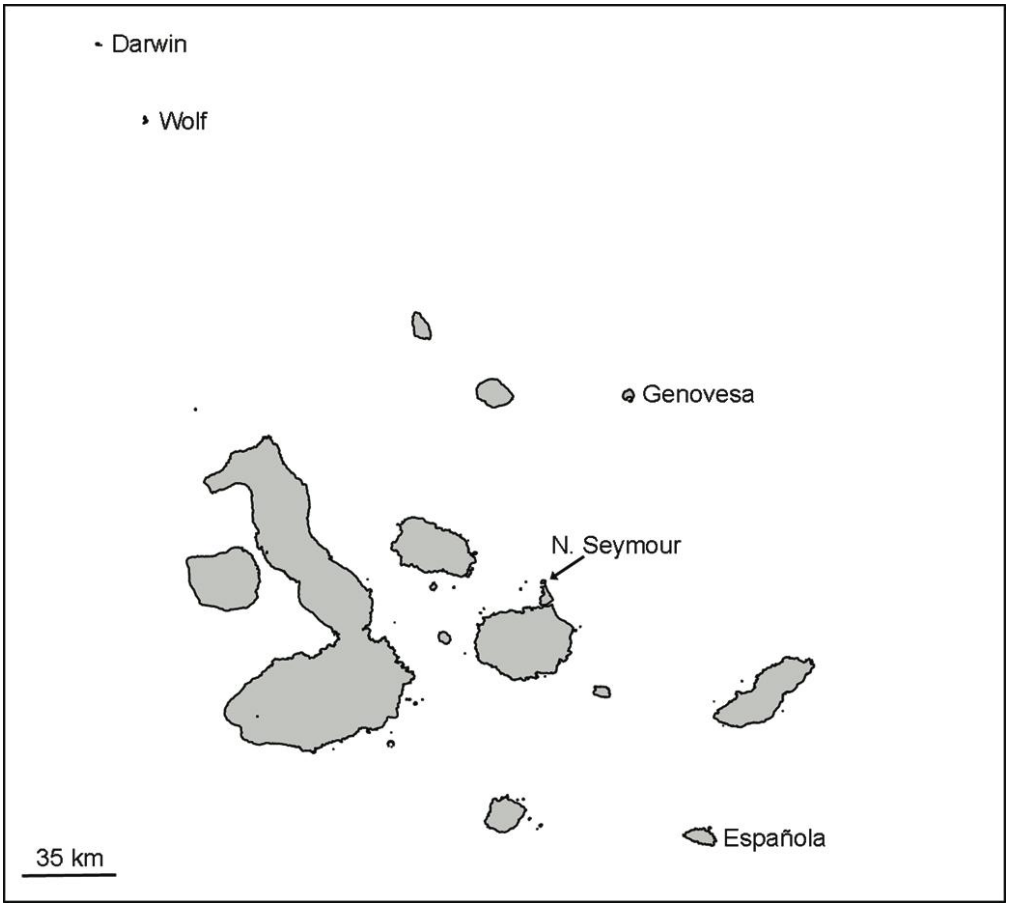
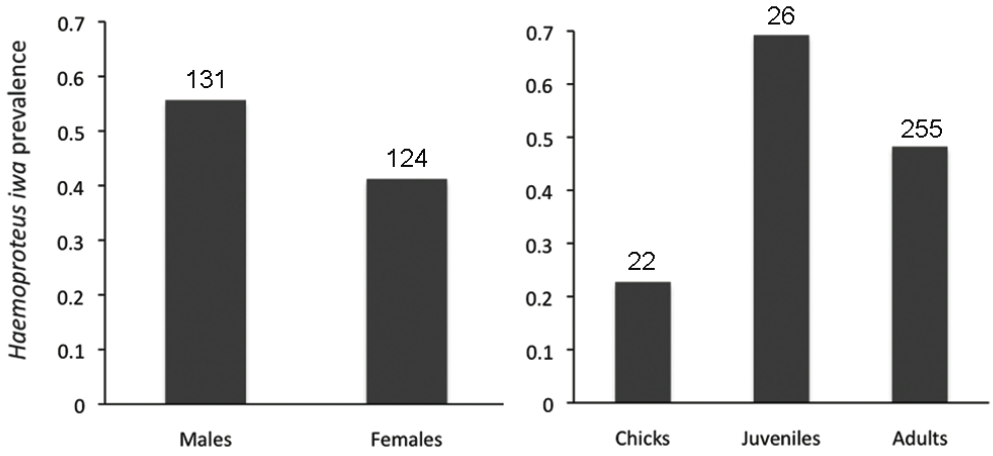


Figure 2


Haemoproteus iwa prevalence
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
131
124
Males
Females
26
22
255
Chicks
Juveniles
Adults

Chapter VII: Population genetics of Nazca Boobies (*Sula granti*) and Great Frigatebirds (*Fregata minor*) in the Galapagos Islands

Levin, I. I. and P.G. Parker, unpublished

Abstract: Seabirds are considered highly mobile, able to fly great distances with few apparent barriers to dispersal. However, it is often the case that seabird populations exhibit strong population genetic structure despite their potential vagility. Here we show that Galapagos Nazca Booby (*Sula granti*) populations are substantially differentiated, especially given the small geographic scale, while Galapagos Great Frigatebird (*Fregata minor*) populations are not. We characterized the genetic differentiation by sampling five colonies of both species in the Galapagos archipelago and analyzing eight microsatellite loci and three mitochondrial genes. Using an F-statistic approach, we found significant differentiation between nearly all island pairs of Nazca Booby populations and a Bayesian clustering analysis provided support for three genetic clusters. One cluster included individuals sampled from the remote, northwestern islands of Darwin and Wolf; a second cluster included individuals sampled from the most eastern site in the archipelago on San Cristobal; and the third cluster included individuals from the northeastern island of Genovesa and the southeastern island of Española. There was no convincing pattern of isolation by distance and seven of nine of the migration rates higher than 0.01 were in the south or southeast to north or northwest direction. The population differentiation in Galapagos Nazca Boobies, but not Great Frigatebirds, is most likely due to strong natal philopatry, as suggested by other recent studies.

Introduction

Island archipelagos have played an important role in our understanding of diversification and speciation. Despite low species diversity, the Galapagos Islands have an exceptionally large proportion of endemic species across flora and fauna (Tye et al., 2002), which has supported a substantial body of research on the processes related to inter-island or inter-population variation and differentiation. The Galapagos are located on the equator, approximately 1000 km off the coast of South America and have never been connected to the mainland. The isolation of the archipelago, and the defining features of island systems (restricted land mass, clearly defined geographical boundaries) make for a useful system in which to understand how populations are shaped by the evolutionary forces of genetic drift, mutation and selection. Due to their restricted area, islands typically harbor smaller populations than are found on continents, which can lead to a stronger effect of genetic drift. The differentiation resulting from genetic drift can be countered by any homogenization caused by gene flow, common in highly mobile organisms that migrate from their natal sites. Galapagos organisms exhibit high variation with respect to population differentiation: on one end of the spectrum, Galapagos Penguins (*Spheniscus mendiculus*)(Nims et al., 2007) and Galapagos Doves (*Zenaida galapagoensis*) (Santiago-Alarcon et al., 2006) have high levels of gene flow between populations, while land iguanas (*Conolophus* sp.)(Tzika et al., 2008), Galapagos Hawks (*Buteo galapagoensis*)(Bollmer et al., 2005) and Galapagos cormorants (*Phalarocorax harrisi*)(Duffie et al., 2007) show high levels of differentiation between islands.

Within seabirds, one finds an apparent paradox between mobility and philopatry; seabirds are some of the most vagile organisms (e.g., Dearborn et al.,

2003; Weimerskirch et al., 2006), and yet they can be the most reluctant to disperse from natal colonies (e.g., Huyvaert and Anderson, 2004). Seabirds presumably encounter few geographic barriers to dispersal (at least within ocean basins), but indirect (genetic) evidence suggests that population differentiation can be strong in many species (Friesen et al., 2007). In their meta-analysis, Friesen et al. (2007) identified two major drivers of population genetic patterns in seabirds: i) Year-round resident species, or species that had population-specific nonbreeding grounds were more likely to have higher levels of population genetic structure and ii) species occupying polar and temperate zones were less likely to be genetically structured than their tropical counterparts possibly from incomplete lineage sorting due to climate fluctuations.

The Galapagos Islands support large numbers of seabirds, both with pan-tropical distributions (e.g., Great Frigatebird (*Fregata minor*), Blue-Footed Booby (*S. nebouxii*), Red-Footed Booby (*Sula sula*), Magnificent Frigatebird (*F. magnificens*)) as well as endemic species (e.g., Galapagos Petrel (*Pterodroma phaepygia*), Flightless Cormorant). The Great Frigatebird breeds in the Pacific, the South Atlantic and the Indian Oceans. The Nazca Booby (*S. granti*) is a common, resident Galapagos seabird throughout the archipelago that was elevated to species status in 2002 after morphological (Pitman and Jehl, 1998) and genetic (Friesen et al., 2002) evaluation demonstrated marked differences from individuals belonging to other Pacific subspecies. The Nazca Booby has a more restricted range than its sister species, the Masked Booby (*S. dactylatra*), with breeding colonies located primarily on oceanic islands on the Nazca tectonic plate, namely the Revillagigedo Islands in

Mexico, Clipperton and Malepo islands in Colombia, the Galapagos Islands and la Plata Islands in Ecuador (Pitman and Jehl, 1998), and records from the Lobos de Afuera Islands, Peru (Figueroa, 2004) and from Oahu and Tern Island in Hawaii (Vanderwerf et al. 2008).

We used eight variable microsatellite DNA markers and mitochondrial DNA (mtDNA) sequence data to describe the population genetic structure of Galapagos Great Frigatebirds and Nazca Boobies. There is some indication that both sexes of Great Frigatebirds are natally philopatric (Metz and Schreiber 2002) and we know that the Galapagos breeding population is genetically distinct from Great Frigatebirds that breed outside of the archipelago (Hailer unpublished data). Breeding dispersal of Nazca Boobies is very limited (Huyvaert and Anderson, 2004), thus we predict that high natal philopatry will promote population differentiation between Galapagos Nazca Booby colonies on different islands. On the other hand, Great Frigatebird populations are expected to show less population differentiation than the Nazca Booby populations. Due to high vagility of both species and documented rare long-distance dispersal events (Booby: Huyvaert and Anderson, 2004; Frigatebird: Dearborn et al. 2003), we make no prediction regarding geographic distance as an isolating barrier for either species.

Materials and methods

Sample collection

Seabirds were sampled in July 2007, June-July 2008, June 2010, and July 2011 from six islands in the Galapagos (Darwin, Española, Genovesa, North Seymour, San Cristobal, and Wolf, Figure 1). Because only two Nazca Boobies were

captured on North Seymour, these individuals were removed from the analyses. Great Frigatebirds captured on San Cristobal were not breeding at the time of sampling, so we did not include them in the analysis. Sample sizes per island can be found in Table 1 and 2. Birds were captured by hand and 2 drops of blood, collected via brachial venipuncture, were preserved in 500 μL of lysis buffer (Longmire et al., 1998).

Laboratory analyses

DNA was extracted following a standard phenol-chloroform extraction protocol (Sambrook et al., 1989). DNA concentrations were estimated by spectrophotometry and diluted to approximately 20 ng/μL for subsequent genetic analyses. Microsatellite markers developed specifically for Great Frigatebirds were used for this species (Table 2)(Dearborn 2008). Microsatellite primers specific for Nazca Boobies were not available. Therefore, we used a number of published markers developed for related booby species that showed sufficient levels of polymorphism (Table S1)(Faircloth et al., 2009; Taylor et al., 2009; Morris-Pocock et al., 2010). Twenty-five primer pairs were tested, and seventeen were rejected due to monomorphism or poor amplification. Aside from three of the frigatebird primers which were fluorescently labeled (Fmin3, Fmin6, Fmin8), one of the primers in each set (typically the shorter one) had a 5' CAG tag applied (Glenn and Schable, 2005). We added a "pigtail" (GTTT) to the 5' end of the primer lacking the CAG tag to facilitate the addition of adenosine by the taq polymerase (Brownstein, et al., 1996). Details on PCR protocol and fragment analysis can be found in the supplemental information. Genemapper v.4.01 (Applied Biosystems, Life Technologies, Carlsbad,

CA) software was used to analyze the fragment analysis results. All individual genotypes were manually scored, 10% of the total samples were repeated across all loci, and roughly one-third of all homozygotes were re-run to ensure we were not incorrectly assigning genotypes due to allelic dropout.

We amplified fragments of three mitochondrial genes, cytochrome *b* (cyt *b*) (780 bp) and NADH dehydrogenase subunit 2 (ND2)(566 bp) and cytochrome oxidase I (COI) (700-800bp) for all Great Frigatebirds and a subset of the Nazca Boobies (n=48). Primers for cyt *b* were B3 and B6 (T. Birt, unpublished, Morris-Pocock et al. 2010), ND2Metl (Haddrath, unpublished; Hailer et al. 2011) and H5766 (Sorenson et al. 1999) were used to amplify ND2, and the entire COI gene was amplified using L6615 and H8121 (Folmer et al. 1994) followed by sequencing with socoiF1 (Chaves et al. 2008 modified from Herbert et al. 2004) and H6035COI_Tyr (Chaves et al. 2008). Details for the PCR reactions, template cleanup, and sequencing can be found in the supplemental information. DNA sequences were obtained using an Applied Biosystems 3100 DNA analyzer at the University of Missouri – St. Louis using BigDye Terminator v3.1 Cycle Sequencing chemistry.

Population genetic structure analyses

Microsatellite DNA analysis

Deviation from Hardy-Weinberg equilibrium (HWE) was tested for each locus with allele randomizations within populations (1000 permutations) and over all populations (10,000 permutations) in FSTAT v. 2.9.3.2 (Goudet, 2001). Genetic variation for each locus within each population was quantified using number of alleles and genetic diversity (Nei 1973) in FSTAT and HP-RARE (Kalinowski, 2005)

was used to calculate rarefied allelic richness per site-locus combination. We tested for the presence of null alleles using ML-NullFreq (Kalinowski, http://www.montana.edu/kalinowski/Software/MLNullFreq.htm). Deviations from linkage equlibria were tested in Arlequin v.3.5.1.2 (Excoffier et al., 2005) using ln likelihood ratio G-tests. Arlequin was used to estimate pairwise differentiation, F_{ST} (Weir and Cockerham, 1984), between all colony pairs. R_{ST} (Slatkin 1995), a similar estimate that allows for a stepwise mutation model was calculated for all colony pairs in FSTAT. A hierarchical Analysis of Molecular Variance (AMOVA) was run in Arlequin if some population differentiation was found. For the Nazca Boobies, we ran the AMOVAs testing for structure using three groups (Darwin + Wolf; Genovesa + Española; San Cristobal) and two groups (Darwin + Wolf + Genovesa + Española and San Cristobal). If genetic differentiation was detected, a factorial correspondence analysis (FCA) was performed on individual multilocus genotypes using GENETIX v.4.0.5.

Genotype clustering was evaluated using a Bayesian method implemented in STRUCTURE v.2.3.3 (Prichard et al., 2000). The most probable number of populations, k, was determined using the second order rate of change in posterior probabilities between runs of different k as described in Evanno et al. (2005). We performed three runs per k (k=1 through k=8) using the *locprior* setting, the admixture model, correlated allele frequencies, and a burn-in of 200,000 cycles followed by 500,000 additional cycles. We also performed shorter runs using different settings (no-admixture model, runs without the *locprior* setting) to evaluate the importance of model choice. Results were averaged for the runs and the program

DISTRUCT v.1.1 (Rosenberg, 2004) was used to construct a visual output from STRCUTURE using the number of populations with the highest likelihood.

Migration rates were estimated using BAYESASS v.1.3, which evaluates gene flow using a model that does not assume migration-drift equilibrium. Default values were used: 3,000,000 Markov chain Monte Carlo (MCMC) iterations, 1,000,000 burn-in iterations, sampling every 2000 iterations, and initial values of delta for allele frequencies, migration rates and inbreeding set at 0.15. If genetic structure was found, we tested for a relationship between geographic distance and genetic differentiation (isolation by distance) using a Mantel test implemented in the program IBD v.1.52 (Bohonak, 2002) on log-transformed geographic distances and Slatkin's linearized F_{ST} values. Geographic distances between colonies were calculated using Google Earth. We tested for recent population bottlenecks using the software BOTTLENECK v1.2.02 (Cornuet ad Luikard 1997). BOTTLENECK detects recent bottleneck events by comparison of allelic diversity and heterozygosity. Allelic diversity decays faster than the correlated measure of diversity, heterozygosity, after a population has experienced a recent reduction, and therefore, heterozygosity excess can be used to infer recent bottlenecks. BOTTLENECK was run using the parameters for the Infinite Allele Model (IAM) (Maruyama and Fuerst, 1985) and sign tests were used to determine statistical significance.

Mitochondrial DNA analyses

Mitochondrial sequences were assembled and manually checked for quality in Seqman 4.0 (DNASTAR, USA) and aligned using BioEdit v.7.0.9.0 (Hall, 1999). The mitochondrial dataset, containing segments of ND2, cytochrome *b* and COI was

tested for neutrality using Tajima's D (Tajima, 1989) tests implemented in DnaSP v.5.10.01 (Librado and Rozas, 2009). Standard diversity indices (haplotype and nucleotide diversity) were calculated in DnaSP. ϕ_{ST} values for all pair-wise colony comparisons were calculated in Arlequin and median joining haplotype networks were calculated in Arlequin and constructed in HapStar (Teacher and Griffiths, 2011).

Results

Diversity within populations

All eight microsatellite loci for both species were found to be in Hardy-Weinberg equilibrium for all populations and no loci showed any signature of null alleles. Overall, we detected 40 alleles in 133 Nazca Boobies (Table 1) and 67 alleles in 114 Great Frigatebirds (Table 2). Allele numbers per locus in Nazca Boobies varied from two to ten (mean = 5) and from two to seventeen (mean = 8.75) in Great Frigatebirds. Seven private alleles were found in Nazca Booby populations, three from the San Cristobal population, three from the Española population and one from the Genovesa population. Ten private alleles were found in Great Frigatebirds, five from the Genovesa population, three from the Española population and from from both Darwin and Wolf. Genetic diversity, measured as number of alleles (N_a), Nei's unbiased genetic diversity (h), and rarefied allelic richness (R_s) varied between different populations (Table 3 for *S. granti*, Table 4 for *F. minor*). In the Nazca Booby populations genetic diversity, h, ranged from 0.071 to 0.870, with a mean of 0.58 and rarefied allelic richness, R_s, ranged from two to eight (mean = 3.8). Average genetic diversity per population was more uniform, ranging from 0.497 in Wolf to 0.572 in Genovesa. Recent population bottlenecks were detected in three of the five

colonies: Española, Genovesa, and San Cristobal. In all three cases, seven of the eight loci showed a relative heterozygosity excess and p-values for the sign tests were 0.042, 0.048, and 0.040 for Española, Genovesa, and San Cristobal respectively. In the Great Frigatebird populations, genetic diversity ranged from 0.283 to 0.926, with a mean of 0.656. Rarefied allelic richness ranged from 2 to 14 (mean = 6.02) and average genetic diversity per population was even, ranging from 0.64 in the North Seymour and Darwin populations to 0.68 in the Wolf population. No recent bottlenecks were detected in Great Frigatebird populations.

A total of 19 mitochondrial haplotypes were detected in Nazca Booby samples using 2,145 bps of mitochondrial DNA sequenced from 48 individuals. Overall haplotype diversity was 0.886 ± 0.028 and overall nucleotide diversity was 0.0011 ± 0.00010. Haplotype and nucleotide diversity per population were very similar, and are shown in Table S2 in the supplementary data and the haplotype network is shown in Figure S1. Tests of neutrality indicated that these DNA regions are evolving in a neutral or nearly-neutral fashion (Tajima's $D = -1.0$, $p > 0.05$). Eighteen haplotypes were identified in Great Frigatebirds, using 1,954 bps of mitochondrial sequence from 108 individuals. Haplotype diversity was 0.644 ± 0.051 while nucleotide diversity was 0.00054 ± 0.00048. Haplotype and nucleotide diversity per population and the mitochondiral haplotype network can be found in Table S3 and Figure S2 respectively. The Tajima's D test gave no indication of non-neutrality ($D = -1.64$, $p > 0.05$).

Differentiation between populations

Using microsatellite loci, we estimated global F_{ST} and R_{ST} for Nazca Booby populations to be 0.070 and 0.071 respectively. Due to the similarity of values given by both F_{ST} and R_{ST}, we will only report and discuss F_{ST} values for all subsequent comparisons. Eight of the ten pair-wise comparisons between colonies using microsatellites were statistically significant ($p < 0.01$) (Table 3). The only colony pair comparisons that did not show significant differentiation using this approach were Darwin and Wolf ($F_{ST} = 0.012$), and Española and Genovesa ($F_{ST} = -0.0003$). In the subsample of mitochondrial DNA sequences, the global ϕ_{ST} was 0.127 and four of the ten pair-wise comparisons between colonies were statistically significant (Table 3). All four significant pair-wise comparisons were between Darwin and all other colonies.

In contrast, the global F_{ST} for Great Frigatebird populations was 0.007. Only two of the ten pair-wise comparisons between island colonies (North Seymour – Wolf, Darwin – Wolf) were statistically significant (Table 4), while most of the comparisons indicated high levels of gene flow between the population pairs. The mitochondrial dataset also showed weak to no genetic structure with a global ϕ_{ST} of 0.023 and only two significantly differentiated population pairs (North Seymour – Darwin, North-Seymour – Wolf).

Further analyses were run only on the Nazca Booby dataset where genetic structure was detected. The FCA analysis showed one population, San Cristobal, clustering separately from other populations, which is in agreement with the other statistical approaches using the multilocus data (Figure S3). The hierarchical

AMOVA run on multilocus data showed strong support for two genetic groups (San Cristobal and all other islands) with 9.52% of the variance among groups and 2.3% of the variance among populations within groups (AMOVA, p = <0.001). When an AMOVA was run with three defined groups (Darwin + Wolf; Genovesa + Española ; San Cristobal), there was marginal support for this structure (AMOVA; p = 0.06). Under this scenario, 8.77% of the variance was among groups and 0.15% was among populations within groups. There was no relationship between F_{ST} and geographic distance using multilocus data (Mantel test: $r^2 = 0.082$, p = 0.07). We did, however, detect a significant positive relationship between geographic distance and ϕ_{ST} values for the mitochondrial data set, but the relationship explained a only a very small amount of the variance and is likely driven by the significant differentiation between Darwin, a peripheral island, and all other colonies (Mantel test: $r^2 = 0.144$, p = 0.02).

The Bayesian clustering analysis performed in STRUCTURE revealed no genetic subdivision in Great Frigatebird populations. In the case of Nazca Booby populations, three were calculated as most likely. One population consisted of the individuals sampled from the isolated, north-western islands of Darwin and Wolf, another included the birds from Española and Genovesa, and the third population consisted of the birds from San Cristobal (Figure 2).

Migration rates for Nazca Boobies calculated in BayesAss had a mean of 0.037 ± 0.072 SD between all pairs of island comparisons. Rates ranged from 0.0029 (95% CI: $2.74e^{-7}$, 00.0182) in the case of movement from Darwin to San Cristobal to 0.2912 (95% CI: 0.2249 – 0.3265) from Española to Genovesa. Seven of the nine migration rates larger than 0.01 were either in the southeast to northwest direction or

south to north direction, with only two north to south or northwest to southeast migration rates greater than 0.01 (from Darwin to Wolf and from Wolf to Genovesa). Migration rates calculated for Great Frigatebirds had a mean of 0.042 ± 0.035. Rates ranged from 0.0065 (95% CI: $1.58e^{-5}$, 0.031) in the case of movement from Darwin to North Seymour to 0.2963 (95% CI: 0.2493, 0.3263) from Española to North Seymour. Migration rates of Great Frigatebirds did not have any clear directional pattern.

Discussion

Our analyses reveal that despite short geographic distances between several of the breeding colonies of Nazca Boobies, there is substantial genetic differentiation within the Galapagos archipelago and that three genetically distinct populations occur within the archipelago, based on the Bayesian clustering analysis. In contrast, very weak to no population genetic structure was found in the Great Frigatebird. Overall, there was only weak signature of isolation by distance among Nazca Booby populations. High levels of nearly unidirectional geneflow were detected between two Nazca Booby colonies, Española and Genovesa. We found that several of the higher migration rates, calculated from the multilocus data, were from Española to other colonies, indicating that it might be a source population. The pronounced genetic differentiation in Galapagos Nazca Boobies detected here corroborates previous mark-recapture studies that demonstrated limited natal and breeding dispersal of Galapagos Nazca Boobies (Huyvaert and Anderson, 2004).

Diversity within populations

Genetic diversity estimates within each population and across all populations were reasonably high and even for both species across populations. Our estimate of 58% (Nazca Booby) and 65% (Great Frigatebird) heterozygosity is similar to values reported for other Galapagos taxa such as the Galapagos Dove (56-65%)(Santiago-Alarcon et al. 2006) and the Flightless Cormorant (51-66%) (Duffie et al., 2009) and higher than Galapagos Penguins (44%) (*Spheniscus mendiculus*) (Nims et al., 2008) and Galapagos Mockingbirds (*Mimus* spp.)(35%)(Hoeck et al., 2010). The caveat when comparing genetic diversity calculated from microsatellites between studies is that ascertainment bias can result from investigators selecting for polymorphic loci during primer development (Ellegren et al., 1995). Additionally, when microsatellites are used for species other than the one they were designed for (as is our case for Nazca Boobies but not Great Frigatebirds), this ascertainment bias can lead to artificial differences due to lower polymorphism in the non-focal species (Brandström and Ellegren, 2008). When compared to the allele numbers indentified in population genetic studies on species for which the markers were developed, one of three we used here had more alleles in the Nazca Booby. The remaining five could only be compared to a small number of individuals as part of the original descriptions of the loci. In these cases, we revealed more alleles in two markers, however we examined 133 individuals while 30 were used for the initial marker description.

Evidence for recent bottlenecks were detected in the Española, Genovesa and San Cristobal Nazca Booby colonies. This could be due to the El Niño Southern Oscillation (ENSO) events that raise sea surface temperature, which can negatively

affect marine life in Galapagos. The 1986-1987 ENSO event, while less severe than the one in 1982-1983, caused Nazca Boobies to either suspend breeding or adjust the timing of their breeding cycle (Anderson et al. 1989).

Haplotype diversities estimated from mitochondrial DNA were fairly high (h = 0.886 for Nazca Boobies, 0.644 for Great Frigatebirds), especially compared to recent colonists like the Galapagos Flycatcher (*Myiarchus magnirostris*) (h = 0.491) that is estimated to have arrived in Galapagos 850,000 years ago (Sari and Parker, in press) and the Galapagos Hawk (*Buteo galapagoensis*) (h = 0.671), which colonized the islands less than 300,000 years ago (Bollmer et al., 2006). Four island colonies of Nazca Boobies had three or more unique mtDNA haplotypes (Darwin = 3, Genovesa = 3 San Cristobal = 4, Wolf = 4), and the most genetically distinct island was Darwin. For Great Frigatebirds, all island populations except Darwin had at least two unique mtDNA haplotypes and there were four haplotypes that were shared between Darwin and Española.

Differentiation between populations

As predicted, population differentiation was more pronounced among Nazca Booby populations compared to populations of Great Frigatebirds. Great Frigatebirds showed very weak to no genetic structure, with the largest F_{ST}, 0.0396, between Darwin and Wolf, the two islands closest in proximity. Even with the *Locprior* setting in STRUCTURE, we detected no population subdivision. Although we have evidence that Galapagos Great Frigatebirds are geneticially distinct from their non-Galapagos conspecifics (Hailer unpublished data), the birds breeding within the archipelago appear to be exchanging genes at a rate that erases any effects of

population differentiation. Aside from lower natal and breeding philopatry, another explanatory factor could be lack of philopatry to non-breeding site. Friesen et al. (2007) found philopatry to non-breeding site to be a strong predictor of population genetic structure. We lack information regarding whether Galapagos Great Frigatebirds use the same non-breeding sites each year.

Nazca Boobies showed pronounced genetic differentiation. As predicted, population differentiation, as measured by F_{ST} calculated with the multilocus dataset, was statistically significant between all but two Nazca Booby population pairs (Genovesa- Española; Darwin-Wolf). The gene flow between Darwin and Wolf is not surprising given that they are separated by only 38 km. Geneflow between Genovesa and Española, separated by 194 km, but not between San Cristobal and either Genovesa (140 km) or Española (87 km) is a bit more puzzling. The FCA analysis also identified San Cristobal Nazca Boobies as very distinct from other colonies (Figure S3). The western tip of San Cristobal is slightly east of a straight line between Española and Genovesa, but the main seabird colonies are located on the extreme northeastern tip of the island, also the most eastern point in the archipelago, with other smaller colonies along islets on the north side. Española birds dispersing in a north-northwestern direction, and therefore not passing over the colony on San Cristobal, would explain our estimates of archipelago-wide directional migration rates, and suggests that most geneflow occurs in a northern or northwestern direction. Interestingly, geneflow was also highest between Galapagos Doves sampled on Española and Genovesa, although San Cristobal was omitted from the analyses due to small sample size (Santiago-Alarcon et al., 2006). Similarly, Arbogast et al., (2006)

found that Galapagos Mockingbirds from Española, Genovesa and San Cristobal had very similar mtDNA despite being considered different species.

Mitochondrial ϕ_{ST} values for Great Frigatebirds were in agreement with the multilocus dataset, showing at most only weak structure. Mitochondrial ϕ_{ST} values for Nazca Boobies were slightly lower than F_{ST}'s calculated with microsatellites for most colony pairs except for Darwin and all other colonies, which showed high levels of differentiation. Although this pattern was not seen in the microsatellite analysis of pair-wise differentiation, we find a similar pattern of differentiation in the extreme corners of the archipelago: Darwin is the most northern and most western of the islands while San Cristobal is the most eastern island currently above sea level. This pattern is evident in Darwin's finches (*Geospiza*, *Camarhynchus*, *Catospiza*, and *Certhidea* spp.), where peripheral populations were found to be more genetically distinct (Petren et al., 2005). However, the larger Nazca Booby colonies we sampled for this study are all arguably peripheral, so it is difficult for us to provide much support for the claim that peripheral isolation is driving this pattern of population differentiation in our system. Finally, despite the fact that, depending on the molecular markers used, different colonies emerge as the most genetically distinct, there are consistencies between the mtDNA and the multilocus datasets. Overall magnitudes of the test statistics differ, but several of the pair-wise relationships tell the same story for both marker types (e.g., Española and Genovesa, Darwin and San Cristobal, Darwin and Española).

No strong relationship was found between geographic distance and genetic differentiation of Nazca Boobies using either mtDNA or microsatellite data. A

Mantel test did detect a significant isolation by distance relationship using ϕ_{ST}, but it appeared to be an artifact of a few points, only explaining 14% of the variation in the data. A positive relationship between geographic distance and genetic differentiation was found in Galapagos passerine birds (Petren et al., 2005; Hoeck et al. 2010) and in the Flightless Cormorants (Duffie et al. 2009) where distance-limited dispersal is not surprising; however, it is not surprising that we do not find isolation by distance effects in a vagile seabird on such a small geographic scale.

The Bayesian clustering analysis detected three distinct populations of Galapagos Nazca Boobies: San Cristobal, Genovesa and Española, and Darwin and Wolf. These results are consistent with the genetic uniqueness of San Cristobal birds (this population, along with Genovesa, had the greatest number of private alleles), and the relative isolation of Darwin and Wolf compared to any other islands in the archipelago. The geneflow between Genovesa and Española is somewhat difficult to explain as mentioned above, but seems to be a recurring theme in other Galapagos birds. Migration rate estimates indicate that the highest level of geneflow occurs from Española to Genovesa and from Wolf to Darwin. Interestingly, there is negligible geneflow from Genovesa to Española. The majority of migration rate estimates greater than 0.01 are in a north or northwestern direction, the direction of the prevailing winds.

Conclusions

Galapagos Nazca Booby colonies are strongly genetically structured given the small geographic scale while Great Frigatebirds are not. Regarding the structure detected in the Nazca Booby, some *Sulidae* species show strong phylogeographic

signals and/or population genetic structure (e.g., Brown Booby (Morris-Pocock et al., 2011); Red-Footed Booby (Morris-Pocock et al., 2010)), while others do not (e.g., Blue-Footed Booby (Taylor et al., 2011a); Peruvian Booby (Taylor et al., 2011b)). A possible explanation for the lack of structure in the Blue-Footed and Peruvian Booby populations is their specialization to cold-water upwelling environments such as the Humboldt Current system. When ENSO events disrupt the upwelling, successful reproduction and survival could depend on movement of individuals to more suitable breeding colonies (Taylor et al., 2011b). Population differentiation in the Galapagos Nazca Booby and other *Sulidae* is most likely due to strong natal philopatry. Median natal dispersal distances for Española Nazca Boobies were 105 m for females and 26 m for males (Huyvaert and Anderson, 2004). Only one breeding dispersal distance within the Punta Cevallos, Española colony was greater than 25 m (Huyvaert and Anderson, 2004). Documented natal dispersal from Española to other Nazca Booby colonies was rare, with an estimate of 1.3% of banded nestlings moving to other surveyed islands (excluding Darwin and Wolf) (Huyvaert and Anderson, 2004). This value is lower than our estimated mean migration rate across the archipelago, 0.037, but that is not surprising given that mark-recapture techniques are sure to miss some natal dispersal events leading to an underestimate. Seventeen band records were reported outside of Galapagos, indicating Galapagos Nazca Boobies can disperse long distances, but will only do so rarely (Huyvaert and Anderson, 2004). These data, and our findings, clearly illustrate what has been called "the seabird paradox" (Milot et al., 2008); where some pelagic species show strong population genetic differentiation despite being highly mobile (Friesen et al. 2007). This paradox raises important

questions involving natal and breeding dispersal, benefits of philopatry and coloniality, potential barriers (physical and non-physical) to dispersal, and colony persistence that are fundamental to our understanding of evolution in seabirds.

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Table 1: Total number of alleles (N_a), Nei's unbiased gene diversity (h), and rarefied allelic richness (R_S for each colony and locus, R_T for all colonies combined) for populations of Galapagos Nazca Boobies (*Sula granti*). Sample size = 133; sample sizes per island: Darwin = 12, Española = 51, Genovesa = 27, San Cristobal = 29, Wolf = 14.

	Darwin			**Española**			**Genovesa**			**San Cristobal**			**Wolf**			**Total**		
Locus	**N_a**	**h**	**R_S**	**N_a**	**h**	**R_S**	**N_a**	**h**	**R_S**	**N_a**	**h**	**R_S**	**N_a**	**h**	**R_S**	**N_a**	**h**	**R_T**
53	3	0.638	3.00	4	0.649	3.24	3	0.649	3.00	3	0.603	3.00	4	0.585	3.86	5	0.727	3.4
83	4	0.649	4.00	6	0.640	4.68	5	0.720	4.68	4	0.662	3.89	4	0.704	4.00	7	0.737	4.6
123	2	0.391	2.00	2	0.503	2.00	2	0.492	2.00	2	0.506	2.00	2	0.519	2.00	2	0.501	2.0
47	2	0.228	2.00	2	0.318	2.00	2	0.372	2.00	2	0.373	2.00	2	0.138	1.98	2	0.316	2.0
110	2	0.083	2.00	3	0.148	2.23	3	0.352	2.93	3	0.222	2.60	2	0.071	1.86	3	0.194	2.6
48	3	0.518	3.00	4	0.646	3.23	3	0.570	2.95	3	0.612	3.00	3	0.553	2.86	4	0.602	3.2
D07	4	0.772	4.00	7	0.698	4.80	6	0.636	4.89	4	0.552	3.22	4	0.590	3.98	7	0.704	5.0
G03	8	0.870	8.00	7	0.758	5.66	9	0.788	7.02	8	0.822	6.83	6	0.817	5.84	10	0.810	7.6
All loci	28			35			33			29			27			40		
Mean		0.519	3.50		0.545	3.48		0.572	3.68		0.544	3.32		0.497	3.30	5	0.585	3.8

Table 2: Total number of alleles (N_a), Nei's unbiased gene diversity (h), and rarefied allelic richness (R_S for each colony and locus, R_T for all colonies combined) for populations of Galapagos Great Frigatebirds (*Fregata minor*). Sample size = 114; sample sizes per island: Darwin = 15, Española = 29, Genovesa = 27, North Seymour = 28, Wolf = 15.

	Darwin			**Española**			**Genovesa**			**North Seymour**			**Wolf**			**Total**		
Locus	**N_a**	**h**	**R_S**	**N_a**	**h**	**R_S**	**N_a**	**h**	**R_S**	**N_a**	**h**	**R_S**	**N_a**	**h**	**R_S**	**N_a**	**h**	**R_T**
Fmin1	6	0.671	6	7	0.685	5.96	7	0.620	5.94	6	0.720	5.86	6	0.690	6	8	0.671	5.95
Fmin4	2	0.333	2	6	0.429	4.92	5	0.363	4.56	4	0.283	3.62	4	0.402	4	7	0.358	3.82
Fmin11	5	0.452	5	3	0.448	2.96	3	0.402	2.99	3	0.436	2.95	4	0.562	4	5	0.451	3.58
Fmin6	5	0.679	5	8	0.771	7.23	8	0.813	7.26	8	0.800	7.41	7	0.798	7	9	0.787	6.78
Fmin18	7	0.790	7	6	0.735	5.28	8	0.822	6.99	7	0.759	5.53	7	0.771	2	8	0.775	5.36
Fmin8	2	0.400	2	2	0.491	2.0	2	0.503	2.0	2	0.420	2.0	2	0.457	7	2	0.492	2.00
Fmin10	10	0.881	10	10	0.792	8.22	8	0.793	7.45	10	0.818	8.44	7	0.821	10	11	0.810	8.82
Fmin2	10	0.890	10	13	0.909	11.1	13	0.907	11.0	14	0.892	10.9	14	0.926	14	17	0.899	11.4
All loci	54			55			54			54			51			67		
Mean		0.637	5.88		0.658	5.96		0.653	6.02		0.641	5.84		0.678	6.75	8.4	0.656	6.09

Table 3: Pair-wise F_{ST} values for Nazca Boobies (*Sula granti*) from microsatellites (n=133) above the diagonal and pair-wise ϕ_{ST} values from mtDNA (n=48) below the diagonal.

	Darwin	**Española**	**Genovesa**	**San Cristobal**	**Wolf**
Darwin		0.033*	0.048*	0.146*	0.012
Española	0.239*		-0.0003	0.108*	0.049*
Genovesa	0.263*	0.070		0.101*	0.050*
San Cristobal	0.302*	-0.019	-0.042		0.164*
Wolf	0.184*	0.032	0.042	0.080	

* denotes F_{ST} and ϕ_{ST} values with p-values < 0.01

Table 4: Pair-wise F_{ST} values for Great Frigatebirds (*Fregata minor*) from microsatellites (n=114) above the diagonal and pair-wise ϕ_{ST} values from mtDNA (n=108) below the diagonal.

	Darwin	**Española**	**Genovesa**	**North Seymour**	**Wolf**
Darwin		0.004	0.017	-0.004	0.040*
Española	0.039		-0.002	-0.006	0.009
Genovesa	0.034	-0.028		0.010	0.007
North Seymour	0.111*	0.018	0.010		0.027*
Wolf	-0.018	0.002	0.002	0.059*	

* denotes F_{ST} and ϕ_{ST} values with p-values < 0.01

Figure Legends

Figure 1: Map of the Galapagos Islands with the islands included in the Nazca Booby (*Sula granti*) analysis labeled. Numbers next to arrows are pairwise F_{ST} values calculated for all island colonies of *S. granti* using eight microsatellite loci. Arrows show directional migration (rates calculated in BayesAss). Thick arrows indicate higher migration rates (0.18-0.29) while thinner arrows represent lower migration rates (0.01-0.06). Lines with no arrowheads have directional migration rates less than 0.01.

Figure 2: Posterior probability of assignment f or 133 Nazca Boobies (*Sula granti*) to three genetic clusters based on a Bayesian analysis run in STRUCTURE of variation at eight microsatellite loci. Individuals are grouped by population and the different genetic clusters are indicated by the different shades of gray.

Figure 1

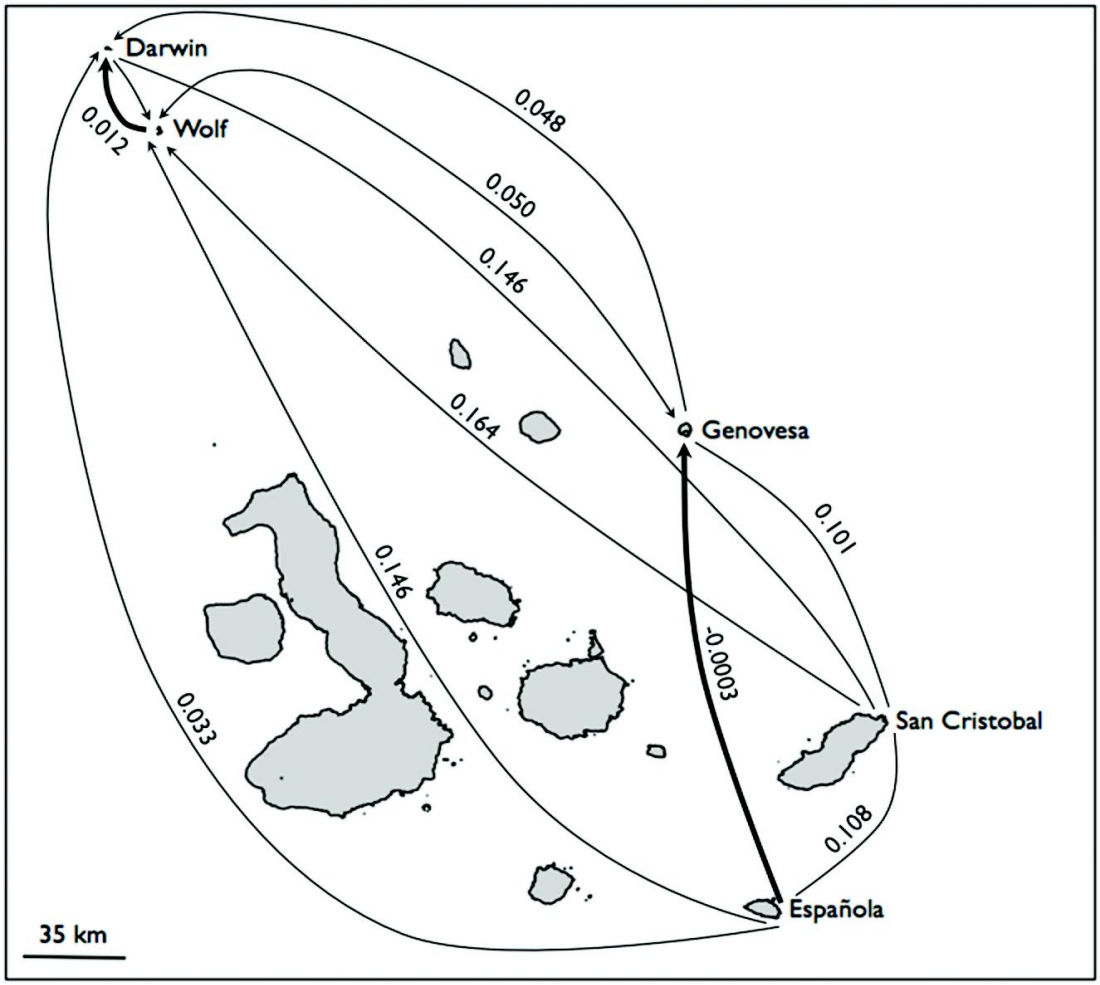
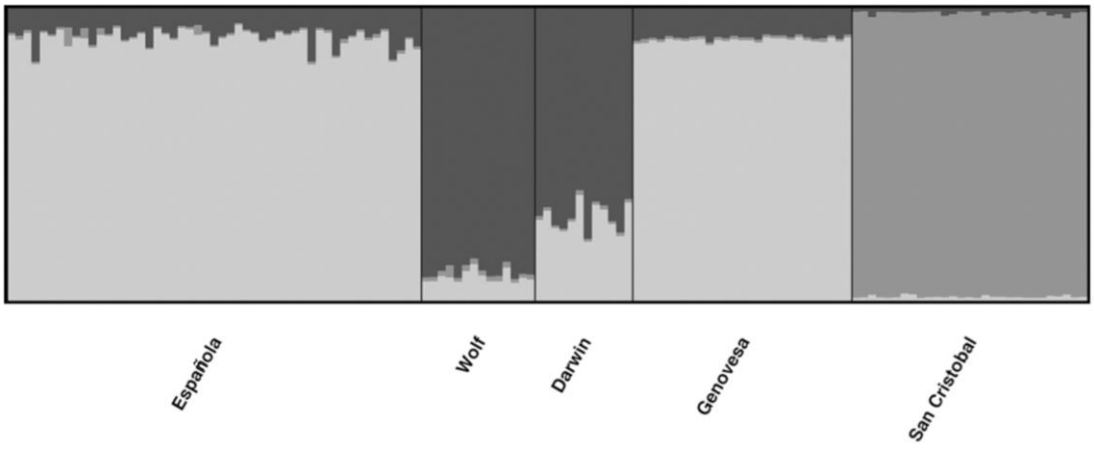

Darwin
Wolf
Genovesa
San Cristobal
Española
0.012
0.048
0.050
0.146
0.164
0.101
0.146
-0.0003
0.033
0.108
35 km

Figure 2



Supplemental information

Microsatellite PCR and fragment analysis

Ten microliter PCR reactions were run using Bioline Red taq polymerase and accompanying reagents (Bioline, Tauton, MA). Reaction conditions for PCR with primers from Taylor et al. (2010) and Morris-Pocock et al. 2010 were: an initial dwell at 94°C for 2 minutes, followed by 16 cycles of 94°C for 45 seconds, 60°C for 45 seconds decreasing by 0.5°C per cycle, and 72°C for 30 seconds. Twenty-one cycles of 94°C for 45 seconds, 52°C for 45 seconds and 72°C for 30 seconds followed the touchdown cycles, as well as one final extension at 72°C for 10 minutes. Reaction conditions for PCR using primers from Faircloth et al. (2009) were: initial dwell at 95°C for 5 minutes, followed by 20 cycles of 95°C for 20 seconds, 65°C for 30 seconds decreasing by 0.5°C per cycle and 72°C for 90 seconds. Twenty cycles of 95°C for 20 seconds, 55°C for 30 seconds and 72°C for 90 seconds followed the touchdown cycles. The protocol ended with a 10 minute final extension at 72°C. The only deviation from the aforementioned reaction chemistry was the addition of BSA to reactions using ss2b-48. Reaction conditions for primers published in Dearborn et al. (2003) follwed the published protocol. Microsatellites were amplified separately and then combined in two multiplex reactions with a size standard, GS500(-250)LIZ (Applied Biosystems (ABI), Life Technologies, Carlsbad, CA), to be read by the ABI 3100 Genetic Analyzer at the University of Missouri – St. Louis.

mtDNA PCR

Mitochondrial DNA PCR reactions (25 microliters) were performed using the following programs. PCR conditions using cyt b primers were: initial dwell at 95°C for 3 minutes, followed by 35 cycles of 95°C for 30 seconds, 49°C for 45 seconds and 72°C for 1 minute. The program completed with a five minute final extension of 72°C. PCR conditions for ND2 were as follows: initial dwell at 95°C for 2 minutes, followed by 45 cycles of 95°C for 40 seconds, 52°C for 40 seconds, and 72°C for 45 seconds. One 10 minute final extension at 72°C completed the program. COI PCR reactions follow Chaves et al. (2008) using the published 63°C annealing temperature for *S. granti* and 62°C for *F. minor*. Reactions were performed using Takara Ex taq polymerase and accompanying reagents (Takara Bio Inc., Japan). PCR products were purified using Exonuclease I (#M0289S, New England Bio Labs Inc., Ipswich, MA) and Antarctic Phosphotase (#M0293S, New England Bio Labs Inc.). Sequencing was done at the University of Missouri – St. Louis using an Applied Biosystems 3100 DNA Analyzer with BigDye Terminator v3.1 Cycle Sequencing chemistry.

Supplement tables and figures

Figure Legends

Figure S1: Haplotype network for *Sula granti* based on three mitochondrial genes. Circles are proportional to the number of individuals that share the haplotypes and the colors correspond to different islands. Black = Darwin, blue = Wolf, green = Genovesa, red = Española, purple = San Cristobal

Figure S2: Haplotype network for Fregata minor based on three mitochondrial genes. Circles are proportional to the number of individuals that share the haplotypes and the colors correspond to different islands. Black = Darwin, blue = Wolf, green = Genovesa, red = Española, yellow = N. Seymour.

Figure S3: Factorial correspondence analysis (FCA) of microsatellite data for *Sula granti.* Pink squares are San Cristobal birds.

Table S1: Primers used to amplify microsatellite loci in Galapagos Nazca Boobies (*Sula granti*). Bold bases indicate the addition of the CAG-tag or PIG-tail and the underlined base indicates the start of the primer sequence.

Locus	Primer sequence	Reference/Accession No.
Sv2a-**53**	F: **CAGTCGGGCGTCATCA**TCTGCAGCTCCCATATTTA	Taylor et al., 2010
	R: **GTTT**CCATGACAGAAGAGATACACTG	GU167930
Sn2b-**83**	F: **GTTT**CTGTTAACCAGAGGAAGGA	Taylor et al. ,2010
	R: **CAGTCGGGCGTCATCA**GAAAGAGGGGTCAGAGAAAT	GU167926
Sn2a-**123**	F: **CAGTCGGGCGTCATCA**TAGTTACCACCATGGCTTT	Taylor et al., 2010
	R: **GTTT**CTGAGCAGGAATCAATCTTC	GU167928
Sv2a-**47**	F: **GTTT**GATGTTCCTTCTGGTGACAG	Taylor et al., 2010
	R: **CAGTCGGGCGTCATCA**GCTCTTAATGACCCTAATG	GU167929
Ss2b-**110**	F: **CAGTCGGGCGTCATCA**CCAGAGAGAATTTCCATTGC	Morris-Pocock et al., 2010
	R: **GTTT**CCATCTGTGTTGAAGGGGTA	GU175418
Ss2b-**48**	F: **GTTT**TCAGCCTTGTTATTCAGC	(Morris-Pocock et al., 2010)
	R: **CAGTCGGGCGTCATCA**GTAGTCATTAACAGGATCAGGA	GU175420
RM4-**D07**	F: **CAGTCGGGCGTCATCA**GCCACCCTCAAGCCATTCC	Faircloth et al., 2010
	R: **GTTT**CCAACAGTTCTGCTGCTCAC	FJ587311
RM4-**G03**	F: **CAGTCGGGCGTCATCA**GGCAGCACTCAAGCTGAAGG	Faircloth et al., 2009
	R: **GTTT**CTCAAGGTAGGGCAGGGTC	FJ587472

Table S2: Sample sizes, number of haplotypes, haplotype diversity (*h*) and nucleotide diversity (π) for ~ 2000 bps of mitochondrial DNA from Galapagos Great Frigatebirds (*Fregata minor*) and Nazca Boobies (*Sula granti*).

Species	**Island**	**n**	**Haplotypes**	***h***	***π***
Fregata minor		108	18	0.633	0.00054
	Darwin	15	3	0.257	0.00014
	Española	26	9	0.668	0.00051
	Genovesa	27	7	0.632	0.00056
	N. Seymour	26	10	0.782	0.00081
	Wolf	14	6	0.604	0.00037
Sula granti		50	19	0.886	0.00010
	Darwin	10	5	0.822	0.00077
	Española	10	4	0.644	0.00077
	Genovesa	10	6	0.911	0.00109
	San Cristobal	10	6	0.889	0.00106
	Wolf	10	4	0.933	0.00098

Figure S1

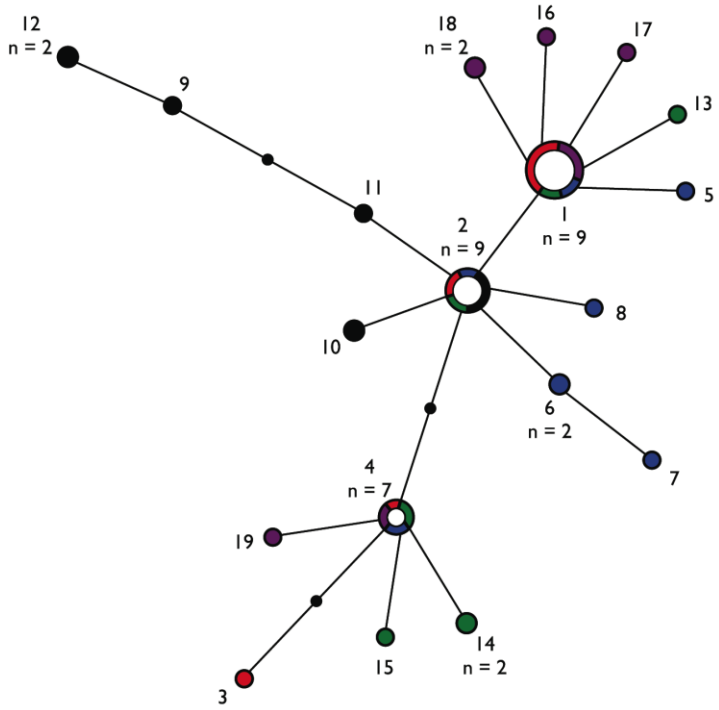


Figure S2

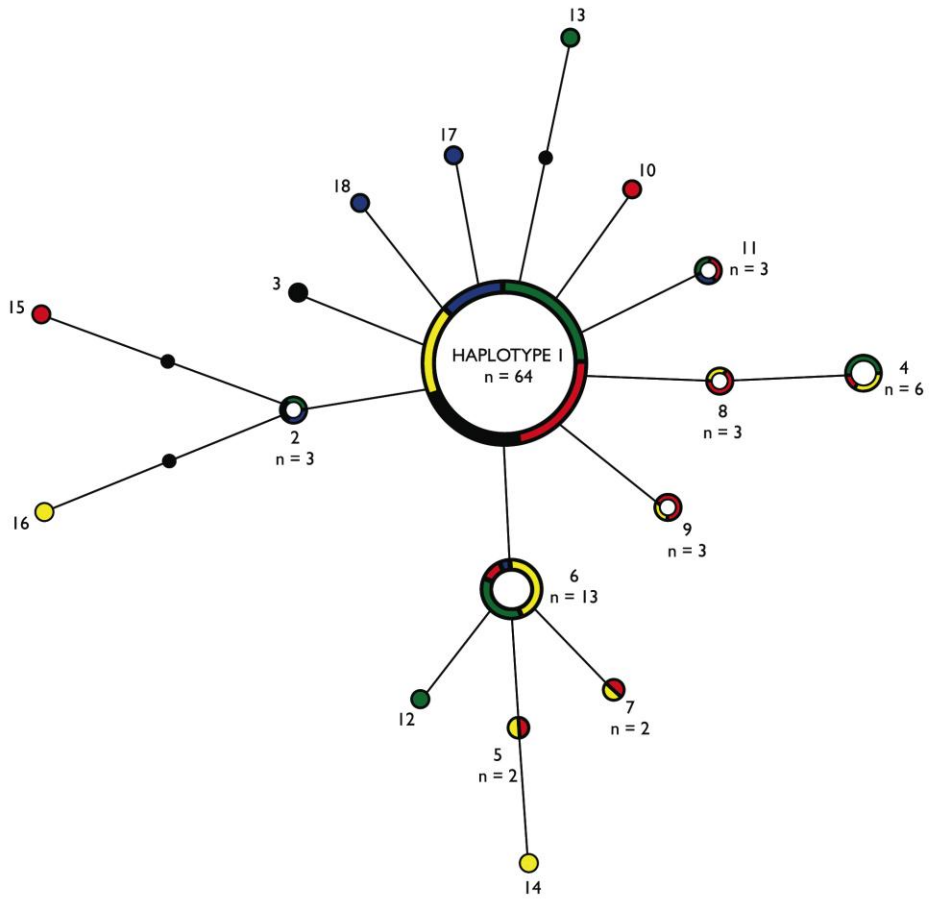
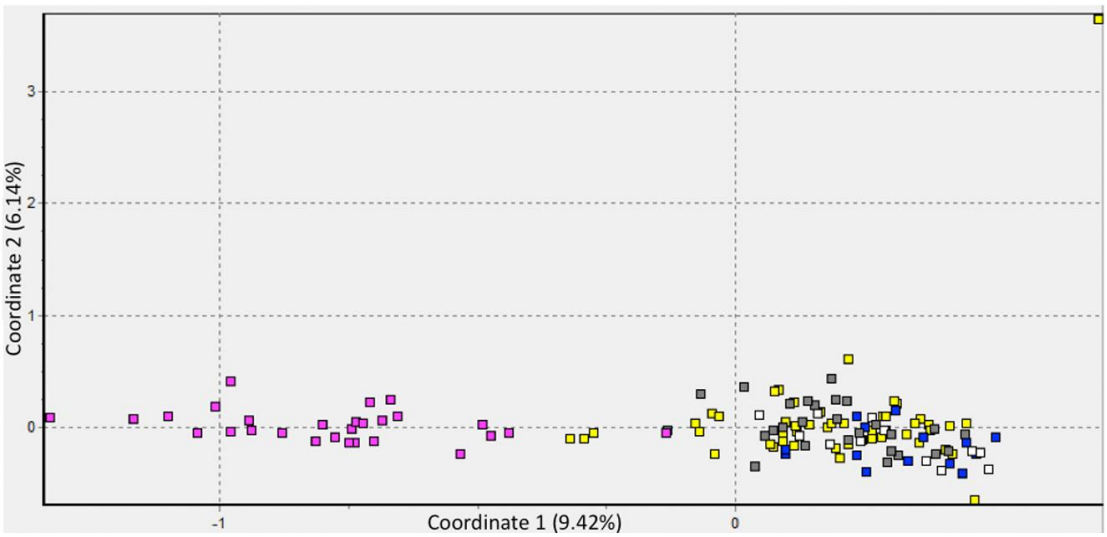

13
17
10
18
11
n = 3
3
15
HAPLOTYPE 1
n = 64
4
n = 6
8
n = 3
2
n = 3
16
9
n = 3
6
n = 13
12
7
n = 2
5
n = 2
14

Figure S3



Chapter VIII: Comparasite host-parasite population genetic structures: Obligate fly ectoparasites on Galapagos seabirds

Levin, I.I. and P.G. Parker, unpublished

Abstract: Host-parasite coevolution is a dynamic process and understanding relative rates of host and parasite gene flow is important for defining the scale at which coevolution might be occurring. Parasites often have larger effective population sizes, shorter generation times and in some cases, faster mutation rates than their hosts, which can lead to greater population differentiation in the parasite relative to the host. However, the opposite is also found; some parasites exhibit less population differentiation than their hosts. Here we present a comparative population genetic study of two seabird species, the Great Frigatebird (*Fregata minor*) and the Nazca Booby (*Sula granti*) and their respective obligate Hippoboscid fly ectoparasites, *Olfersia spinifera* and *O. aenescens*. *Olfersia spinifera* is the vector of a haemosporidian parasite, *Haemoproteus iwa*, which infects frigatebirds throughout their pantropical range. Interestingly, there is no genetic differentiation in the haemosporidian parasite across this range despite strong genetic differentiation between Galapagos frigatebirds and their non-Galapagos conspecifics. It is possible that the broad distribution of this one *H. iwa* lineage could be facilitated by movement of infected *O. spinifera*. Therefore, we predicted more gene flow in both fly species compared to the bird hosts, regardless of the differences in host population genetic structure. Mitochondrial DNA sequence data from three genes per species indicated that despite marked differences in the genetic structure of the bird hosts, gene flow was very high in both fly species. A likely explanation of higher gene flow in both fly species compared to their bird hosts involves non-breeding movements,

including movement of juveniles, prospecting by young and breeding birds, and movement by adult birds whose breeding attempt has failed.

Introduction

Parasites exhibit a wide range of life history strategies that contribute to different dispersal abilities, host specialization, transmission modes, life-cycle complexity and population structure. Population genetic approaches can be used to understand the ecology and evolution of single species and recognizing the impact of host population genetic structure on that of the parasite, comparative studies of interacting species are becoming more common (e.g., McCoy et al. 2005; Whiteman et al. 2007; Bruyndonckx et al. 2009; Jones and Britten 2010; Stefka et al. 2011). This comparative approach is especially important in understanding dispersal rates in hosts and parasites, which are instrumental in defining the scale at which coevolution may be occurring. Coevolution is a dynamic process and variation in gene flow across heterogeneous landscapes can fundamentally alter the outcome of coevolutionary relationships, even within the same system. Forde et al. (2004) and Morgan et al. (2005) used bacteria – bacteriophage systems to demonstrate that gene flow, particularly gene flow in the bacteriophage, across spatially structured landscapes alters the coevolutionary relationship and the resulting patterns of adaptation.

The findings from population genetic analyses of hosts and parasites are as variable as the nature of the interactions themselves. Congruence between host and parasite population genetic structure (or lack of structure) depends on relative rates of host and parasite dispersal, host specificity of the parasite, host and parasite

geographic distribution as well as a myriad of ecological factors that can influence hosts and parasites (Dybdahl and Lively 1996; Johnson et al. 2002; McCoy et al. 2003; Weckstein 2004). Parasites are often cited as having higher evolutionary potential compared to their hosts due to larger effective population sizes, shorter generation times and in some cases, faster mutation rates (Page et al., 1998). In an obligate, host-specific parasite, this could lead to greater population differentiation in the parasite relative to the host. This pattern has been shown across a wide range of host-parasite interactions, from a host plant and fungal pathogen (Delmotte et al. 1999), Black-legged Kittiwake and tick ectoparasite (McCoy et al. 2005), raptor and lice and fly ectoparasites (Whiteman et al. 2007) to butterflies and specialist parasitoids (Anton et al. 2007). However, there are also a number of examples showing the opposite pattern: parasites that exhibit less population differentiation than their hosts (e.g., a freshwater snail and *Schistosoma* parasite, Davies et al. 1999; stinging nettle and its parasitic plant, Mutikainen and Koskela 2002; two shearwater seabirds and their louse and flea ectoparasites, Gomez-Diaz et al. 2007; and prairie dogs and their flea ectoparasites, Jones and Britten 2010). Untangling the factors acting on both hosts and parasites that contribute to these disparate patterns is important for understanding the context of coevolutionary intereactions.

Seabirds provide a good system to investigate population differentiation in hosts and parasites. Seabirds are often very philopatric (Friesen et al., 2007), which can contribute to strong population differentiation despite high potential vagility. Many seabirds are large-bodied, and harbor high numbers of diverse groups of parasites (Hughes and Page 2007). We investigated the population genetic structure

of two seabird species, Great Frigatebirds (*Fregata minor*) and Nazca Boobies (*Sula granti*) and their respective obligate Hippoboscid fly ectoparasites, *Olfersia spinifera* and *O. aenescens* in the Galapagos Islands, Ecuador. There is convincing evidence that *O. spinifera* is the vector of a haemosporidian parasite, *Haemoproteus iwa*, that infects frigatebirds throughout their geographic range (Levin et al. 2011). Interestingly, we have found no genetic differentiation in the haemosporidian parasite across this range despite strong genetic differentiation between Galapagos frigatebirds (*F. magnificens* and *F. minor*) and their non-Galapagos conspecifics (Hailer et al. 2011, Hailer unpublished data). It is possible that the broad distribution of this one *H. iwa* lineage could be facilitated by movement of infected *O. spinifera*. Therefore, we predicted less population genetic structure in *O. spinifera* than in the bird host, *F. minor*. We use *S. granti* and *O. aenescens* as a comparison, because we know from multilocus and mitochondrial data that *S. granti* shows strong population differentiation even at the small geographic scale within the Galapagos islands, while *F. minor* shows weak to no differentiation (Levin and Parker, unpublished data). If Hippoboscid flies are moving between individuals at roosting or non-breeding sites, we expect to find more gene flow in both fly species relative to gene flow in the bird hosts, regardless of the strength host population genetic structure.

Materials and methods

Sampling

We sampled *F. minor*, *S. granti* and their fly ectoparasites from six different islands (Darwin, Española, Genovesa, North Seymour, San Cristobal and Wolf)(Figure 1) in the Galapagos Archipelago during June and July of 2007, 2008,

2010 and 2011. Although breeding is not synchronous throughout the archipelago, there were typically sufficient numbers of breeding adults to sample. Seabirds were captured by hand and a small blood sample was taken from the brachial vein. Blood was preserved in lysis buffer at ambient temperature in the field and later stored at 4°C in the laboratory. Birds were systematically searched for flies and, if present, at least one was collected and stored in 95% ethanol. Once in the lab, flies were kept at -20°C until DNA extraction.

Host DNA extraction and mitochondrial DNA amplification

Bird DNA was extracted from blood using a standard phenol-chloroform extraction protocol (Sambrook et al. 1989), DNA concentrations were estimated by spectrophotometry and diluted to approximately 20 ng/μL for subsequent genetic analyses. Polymerase chain reaction (PCR) was used to amplify regions of the mitochondrial genes cytochrome b (cyt b), cytochrome oxidase I (COI), and NADH dehydrogenase subunit 2 (ND2). Primers for cyt *b* were B3 and B6 (T. Birt, unpublished, Morris-Pocock et al. 2010) and ND2Metl (Haddrath, unpublished; Hailer et al. 2011) and H5766 (Sorenson et al. 1999) were used to amplify ND2. The entire COI gene was amplified using L6615 and H8121 (Folmer et al. 1994) followed by sequencing with socoiF1 (Chaves et al. 2008 modified from Herbert et al. 2004) and H6035COI_Tyr (Chaves et al. 2008). Mitochondrial DNA PCR reactions (25 microliters) were performed using the following programs. PCR conditions using cyt b primers were: initial dwell at 95°C for 3 minutes, followed by 35 cycles of 95°C for 30 seconds, 49°C for 45 seconds and 72°C for 1 minute. The program completed with a five minute final extension of 72°C. PCR conditions for ND2 were as follows:

initial dwell at 95°C for 2 minutes, followed by 45 cycles of 95°C for 40 seconds, 52°C for 40 seconds, and 72°C for 45 seconds. One 10 minute final extension at 72°C completed the program. COI PCR reactions follow Chaves et al. (2008) using the published 63°C annealing temperature for *S. granti* and 62°C for *F. minor*. Reactions were performed using Takara Ex taq polymerase and accompanying reagents (Takara Bio Inc., Japan). Reaction chemistry for all protocols was as follows: PCR products were purified using Exonuclease I (#M0289S, New England Bio Labs Inc., Ipswich, MA) and Antarctic Phosphotase (#M0293S, New England Bio Labs Inc.). Double-stranded sequencing was done at the University of Missouri – St. Louis using an Applied Biosystems 3100 DNA Analyzer with BigDye Terminator v3.1 Cycle Sequencing chemistry.

Parasite DNA extraction and mitochondrial DNA amplification

Thoraxes of hippoboscid flies were separated from heads and abdomens. A Qiagen DNEasy Blood and Tissue DNA extraction kit (Qiagen, USA) was used to individually extract the DNA from each fly thorax. The standard protocol was followed, but DNA was eluted in half as much buffer due to assumed low concentrations of any parasite or host DNA. Undiluted DNA was used in PCR reactions. Cytochrome oxidase I was amplified using LCO1490 and HCO2198 (Folmer et al. 1994) following the reaction conditions described in Whiteman et al. (2006) except for an annealing temperature of 46°C rather than 40°C. A region of mitochondrial 12S ribosomal DNA was amplified using the primer pair 12SAI and 12SBI (Simon et al. 1994) using the reaction conditions found in Whiteman et al. (2006). The primer pair L11122 and H11823 was used to amplify a portion of

cytochrome b following the protocol described in Page et al. (1998). Purification of PCR product and subsequent sequencing was performed as described above.

Population genetic analyses

DNA sequences were assembled and edited in Seqman 4.0 (DNASTAR, USA) and aligned by ClustlW implemented in BioEdit v7.0.5.3 (Hall 1999). All three gene regions were aligned seperately, cropped, concatenated and analyzed together for both hosts and parasites. Population equilibrium and selective neutrality were assessed using a Tajima's D-test (Tajima 1989) in DNASP v.5.10.01 (Librado and Rozas 2009). Minimum spanning haplotype networks were calculated using ARLEQUIN v3.5.1.2 (Excoffier et al. 2005), drawn using HapStar (Teacher and Griffiths 2011) and colored for clarity in Inscape v.0.48.2. Haplotype and nucleotide diversities were calculated in DNASP. We used traditional F-statistics (Wright 1951) to assess variation within and between populations. Analysis of molecular variance (AMOVA, Excoffier et al. 1992) was used to partition components of genetic variation among and within island populations. The number of migrants per generation (N*m*) was estimated from F_{ST} values using Wright's formula (Wright 1951) and used to compare relative amounts of movement between the two bird hosts, the two fly parasites and between the respective bird-parasite pairs. If some level of population genetic differentiation was found, we tested for isolation by distance using Slatkin's linearized F_{ST} ($F_{ST}/(1-F_{ST})$) in the program IBD (Bohonak 2002).

Results

F. minor and O. spinifera population genetic structure

A total of 1,954 bp of mitochondrial DNA (after editing and cropping to equal length) were amplified for *F. minor* (Cyt b: 766 bp, ND2: 489 bp, COI: 699 bp) and 1608 bp were amplified for *O. spinifera* (Cyt B: 630 bp, 12S: 362 bp, COI: 616 bp). There was no indication of non-neutrality in *F. minor* sequence data (Tajima's D = -1.64, P > 0.05) but *O. spinifera* sequences showed a significant departure from neutrality as determined by the Tajima's D test (D = -2.49, p <0.01). Fourteen variable sites were recovered from *F. minor* sequence, seven of which were parsimony informative sites. In comparison, 27 variable sites were found in *O. spinifera*, only seven of which were parsimony informative sites. Sample sizes (total and per island), number of haplotypes, haplotype diversity (*h*) and nucleotide diversity (π) can be found in Table 1. For *F. minor*, the lowest haplotype diversity, 0.257, was found in the birds sampled from Darwin, and the highest was found in the N. Seymour sample (0.783). For the frigatebird fly, *O. spinifera*, the lowest haplotype diversity was recovered from Wolf; however, we only captured two flies from this island. The island with the most diverse *O. spinifera* haplotypes was Genovesa (0.649). Haplotype networks for *F. minor* and *O. spinifera* can be found in Figures 2 and 3.

An analysis of molecular variance showed very weak population genetic structure in *F. minor* (Table 2) with only 2.29% of the variance partitioned among island populations and a global ϕ_{ST} of 0.023. The AMOVA run on the *O. spinifera* dataset showed no support for any subdivision of genetic diversity (p = 0.971). Pair-

wise F_{ST} values for *F. minor* and *O. spinifera* can be found in Table 3. Two pair-wise comparisons (N. Seymour – Darwin, N. Seymour - Wolf) were significant for *F. minor*. No pair-wise comparisons between any *O. spinifera* populations sampled indicated significant differentiation (Table 3). The estimated number of *F. minor* migrants per generation (N*m*) ranged from 4.01 between North Seymour and Darwin to infinitely many between Española and Genovesa and Darwin and Wolf. *Olfersia spinifera* show complete panmixia within Galapagos, with all N*m* estimates indicating infinitely many individuals moving between sites per generation. Wilcoxon signed-rank tests indicated no significant difference between haplotype ($p = 0.63$) and nucleotide ($p = 1$) diversities of *F. minor* and *O. spinifera* populations from the same islands. There was no support for a pattern of isolation by distance between island populations of F. minor (Mantel tests, genetic distance vs. geographic distance: $z = 85.1$, $r = 0.34$. $p = 0.13$; genetic distance vs. log (geographic distance): $z = 0.72$, $r = 0.40$, $p = 0.09$).

S. granti and O. aenescens population genetic structure

We obtained slightly longer COI sequences for *S. granti* (799 bp) giving us a total amount of 2145 bp (Cyt b: 780 bp, ND2: 566). One thousand six-hundred and seventy one base pairs of mitochondrial DNA were used for analyses of *O. aenescens* (Cyt b: 678, 12S: 361. COI: 632). *Sula granti* and *O. aenescens* sequence data showed no departure from neutrality (Tajima's D, *S. granti*: $D = -1.00$, $p > 0.05$; *O. aenescens*: $D = 1.75$, $p > 0.05$). Sample sizes (total and per island), number of haplotypes, haplotype diversity (*h*) and nucleotide diversity (π) can be found in Table 1. Very few flies were captured from *S. granti*; this species, like related Galapagos

sulids, has fewer ectoparasites than Galapagos frigatebirds (Levin, unpublished data). Overall, haplotype diversity in *S. granti* ranged from 0.644 on Española to 0.933 on wolf (Table 2). Average haplotype diversity of *O. aenescens* was 0.830; however, that calculation is based on a small sample including only the three islands that had more than one haplotype sampled. Haplotype networks for *S. granti* and *O. aenescens* can be found in Figures 4 and 5.

Analyses of molecular variance revealed significant genetic differentiation in *S. granti* but not *O. aenescens*; the among population component was a good predictor of genetic partitioning in *S. granti* ($p = 0.00098$), explaining 13.49% of the variance (Table 2), while no differentiation was detected in *O. aenescens* ($p = 0.808$). Four of the ten pair-wise F_{ST}'s (Darwin vs. remaining four islands) were significant in the case of *S. granti*, while no significant pair-wise comparisons were found for *O. aenescens*. Relative number of *S. granti* migrants per generation (N_m) ranged from 1.39 in the case of migrants between Española and Darwin to infinitely many between Española and San Cristobal. *Olfersia aenescens* showed patterns of unrestricted gene flow across all population pairs, with the lowest N*m* estimate of 72.5 between Española and San Cristobal. Haplotype and nucleotide diversities per island population were not significantly different between *S. granti* and *O. aenescens* (Wilcoxon signed-rank test: Haplotype diversity: $p = 0.75$, nucleotide diversity: $p = 0.25$). The genetic structure of *S. granti* populations did have some signature of isolation by distance, driven largely by the significant differentiation between Darwin, a peripheral island, and all other populations (Mantel test, genetic distance vs. geographic distance: $z = 491.84$, $r = 0.38$, $p = 0.02$).

Discussion

Host movement has been shown to be a key determinant of parasite gene flow. However, host movement is often assessed indirectly via population genetic studies that only reveal true dispersal events, where movement is followed by reproduction. By simultaneously applying these same indirect genetic assessments of geneflow to closely-associated parasites, we increase our ability to detect host movement that is not necessarily associated with successful reproduction. Here we show that two obligate fly ectoparasite species have higher levels of gene flow than their respective host species, despite marked differences in the genetic structures of the host populations.

There were no significant differences in genetic diversity measures between either host and parasite pair, but relatively more genetic diversity was partitioned among island populations in the birds than in the flies. This pattern is evident in the haplotype networks (Figures 2-5). Interestingly, both the star-like structure of the *O. spinifera* network and the significant Tajima's D statistic indicate a recent, rapid population expansion of this population. There are a number of possible explanations for this. It is possible that the population of frigatebirds colonizing the Galapagos were free of *O. spinifera*; however, we have rarely handled a frigatebird that does not have at least one fly parasite. We have no reason to believe that non-Galapagos *F. minor* are less parasitized; their large bodies and high survival coupled with their non-diving behavior makes them good hosts for ectoparasites (e.g., Felso and Rozsa 2006). Alternatively, recent expansion could be due to population bottlenecks caused by El Niño Southern Oscillation (ENSO) events that dramatically affect the climatic

conditions in the Galapagos Islands (Valle et al., 1987). We understand little about Hippoboscid breeding biology, but it is possible that the increased precipitation could affect flies in their pupal stage, the only life stage that is off the host. If there is low survuval of pupae and adult flies do not live until the next breeding season (related Hippoboscid flies estimated to live approximately 80 -100 days (Nelson et al. 1975)), this could contribute to a population bottleneck.

It is difficult to imagine that Hippoboscid flies are able to disperse between islands without being attached to a bird host. We do know that, despite being a host-specific, obligate parasite, *O. spinifera* are frequently moving between *F. minor* hosts on a local (within island colony) scale (Levin and Parker, unpublished). It is possible that the larger scale fly movements indicated by these genetic data are facilitated by bird hosts other than the ones we analyzed here; *O. spinifera* also parasitize Magnificent Frigatebirds (*F. magnificens*), which are found breeding on some islands in the Galapagos and *O. aenescens* are reported from other Sulid species such as the Blue-footed Booby (*S. nebouxii*) and the Red-footed Booby (*S. sula*), both of which breed on islands in the Galapagos. Frigatebirds and Booby species are often found nesting in mixed seabird colonies in the Galapagos, but we have not found *O. aenescens* on frigatebirds or *O. spinifera* on booby species. Based only on cyt b sequence divergence, these two fly species differ by 8.5%. Ectoparasite dispersal via alternative hosts has been suggested in the Black-tailed Prairie dogs - flea (*Oropsylla hirsuta*) system where a similar pattern of higher ectoparasite gene flow relative to host gene flow was found (Jones and Britten 2010).

It is possible that *S. granti*'s congeners, *S. sula* or *S. nebouxii*, could be moving *O. aenescens* around the archipelago. At the sites we sampled, there was at least one other species of Sulid breeding (Española: *S. granti* and *S. nebouxii*; Genovesa, Darwin, Wolf: *S. granti* and *S. sula*, San Cristobal: *S. granti*, *S. nebouxii* and *S. sula*). No genetic differentiation was found among *S. nebouxii* populations, based on a comparison of samples from three island colonies (Taylor et al. 2011). A comparison of three colonies of *S. sula* indicated significant differentiation between one pair of the islands (Darwin and Genovesa) (Baiao and Parker, unpublished). It is also possible that *O. aenescens* specialize on the different *Sulidae* species, but whether there is any indication of host race formation has not been tested. There are fewer colonies that have both *F. minor* and *F. magnificens* breeding in close proximity in the Galapagos, making *F. magnificens* movement a less likely explanation for the observed pattern of gene flow between *O. spinifera* collected from *F. minor*.

A likely explanation of higher gene flow in both fly species compared to their bird hosts involves non-breeding movements, including movement of juveniles, prospecting by young and breeding birds and movement by adult birds whose breeding attempt has failed. Frigatebirds are not sexually mature until at least five years of age (Valle et al. 2006) and we do not know the extent of their movements prior to breeding. Even if they are philopatric to their natal site as has been suggested (Metz and Schreiber 2002; Dearborn et al. 2003), movement of juveniles prior to breeding age could facilitate ectoparasite dispersal. Frequent shorter, inter-island and long distance movements of *F. minor* are reported both in the breeding season and

during the non-breeding season (Dearborn et al 2003). Friesen et al. (2007) found that the extent of population genetic structure in seabirds can be explained in part by non-breeding distributions. Philopatry to non-breeding areas appears to reduce or prevent gene flow between seabird populations (Friesen et al. 2007). There is evidence from radio telemetry data on post-breeding movements that suggests Frigatebirds are not always philopatric to non-breeding sites (Weimerskirtch et al 2006). Long-distance dispersal events have been recorded rarely in *S. granti*, with most breeding and natal dispersal distances on the order of 100 m or less (Huyvaert and Anderson 2004).

Theory predicts that gene flow is an important force for introducing novel or lost genetic variation into populations (Gandon et al. 1996) and it has been suggested that greater relative rates of dispersal in parasites compared to their hosts should increase parasite local adaptation (Gandon and Michalakis 2002). Studies of Black-legged Kittiwakes (*Rissa tridactyla*) show that relative gene flow in hosts and parasites (in this case the tick, *Ixodes uriae*) are scale-dependent (McCoy et al. 2002; McCoy et al. 2005). Tick gene flow was similar or higher than kittiwake gene glow at a regional scale, but more restricted at a larger scale (McCoy et al. 2005). Because of the one ubiquitous lineage of haemosporidian parasite, *H. iwa*, in frigatebirds sampled throughout their range (Levin et al. 2011), we hypothesize that the gene flow in Hippoboscid flies demonstrated here could suggest frequent contact between frigatebirds from different breeding colonies on a large geographic scale.

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Figure legends:

Figure 1: Map of the Galapagos Islands with islands included in study in colored boxes. The same colors are used in haplotype networks.

Figure 2: Haplotype network for Galapagos Great Frigatebirds (*Fregata minor*) constructed from mitochondrial DNA. Circles are proportional to the number of individuals that share that haplotype and colors correspond to different islands. Black = Darwin, blue = Wolf, green = Genovesa, yellow = North Seymour, red = Española.

Figure 3: Haplotype network for the Hippoboscid fly, *Olfersia spinifera*, constructed from mitochondrial DNA. *Olfersia spinifera* were collected from Galapagos Great Frigatebirds (*Fregata minor*). Circles are proportional to the number of individuals that share that haplotype and colors correspond to different islands. Black = Darwin, blue = Wolf, green = Genovesa, yellow = North Seymour, red = Española.

Figure 4: Haplotype network for Galapagos Nazca Boobies (*Sula granti*) constructed from mitochondrial DNA. Circles are proportional to the number of individuals that share that haplotype and colors correspond to different islands. Black = Darwin, blue = Wolf, green = Genovesa, purple = San Cristobal, red = Española.

Figure 4: Haplotype network for the Hippoboscid fly, *Olfersia aenescens*, constructed from mitochondrial DNA. *Olfersia aenescens* were collected from Galapagos Nazca Boobies (*Sula granti*). Circles are proportional to the number of individuals that

share that haplotype and colors correspond to different islands. Black = Darwin, purple = San Cristobal, green = Genovesa, yellow = North Seymour, red = Española.

Table 1: Sample sizes, number of haplotypes, haplotype diversity (*h*) and nucleotide diversity (π) for two seabird species, Great Frigatebird (Fregata minor) and Nazca Booby (Sula granti) and their respective Hippoboscid fly ectoparasites (*Olfersia spinifera* and *O. aenescens*).

Species	**Island**	**n**	**Haplotypes**	***h***	***π***
Fregata minor		108	18	0.633	0.00054
	Darwin	15	3	0.257	0.00014
	Española	26	9	0.668	0.00051
	Genovesa	27	7	0.632	0.00056
	N. Seymour	26	10	0.782	0.00081
	Wolf	14	6	0.604	0.00037
Olfersia spinifera (from *F. minor*)		98	26	0.596	0.00057
	Darwin	10	4	0.533	0.00050
	Española	28	11	0.595	0.00062
	Genovesa	22	8	0.649	0.00059
	N. Seymour	36	13	0.629	0.00058
	Wolf	2	1	0	0
Sula granti		50	19	0.886	0.00010
	Darwin	10	5	0.822	0.00077
	Española	10	4	0.644	0.00077
	Genovesa	10	6	0.911	0.00109
	San Cristobal	10	6	0.889	0.00106
	Wolf	10	4	0.933	0.00098
Olfersia aenescens (from *S. granti*)		19	6	0.830	0.00158
	Darwin	1	1	NA	NA
	Española	5	5	1	0.00168
	Genovesa	7	4	0.857	0.00165
	N. Seymour	1	1	NA	NA
	San Cristobal	5	4	0.900	0.00180

Table 2: Summary of AMOVA results for both bird host species.

Species	Partition	d.f.	% variation	ϕ_{ST}	*P*
F. minor	Among-island populations	4	2.29	0.023	0.06
	Within-island populations	103	97.71		
S. granti	Among island populations	4	13.49	0.135	<0.001
	Within island populations	45	86.51		

Table 3: F_{ST} values from mtDNA for Great Frigatebirds (*Fregata minor*) above the diagonal and *Olfersia spinifera* ectoparastic flies below the diagonal. ** = p <0.01. * = p <0.05.

	Darwin	Española	Genovesa	N. Seymour	Wolf
Darwin		0.03852	0.03373	0.11076**	-0.01777
Española	-0.02435		-0.02794	0.01752	0.00249
Genovesa	-0.01638	-0.00482		0.00975	0.00191
N. Seymour	-0.01964	-0.01137	-0.01055		0.05923*
Wolf	-0.32353	-0.32239	-0.28241	-0.31409	

Table 4: F_{ST} values from mtDNA for Nazca Boobies (*Sula granti*) above the diagonal and *Olfersia aenescens* ectoparastic flies below the diagonal. ** = p <0.01. * = p <0.05.

	Darwin	**Española**	**Genovesa**	**San Cristobal**	**Wolf**
Darwin		0.23868***	0.26337***	0.30159***	0.18357**
Española	NA		0.07061	-0.01852	0.03207
Genovesa	NA	-0.01347		0.06504	0.04215
San Cristobal	NA	0.00685	-0.18443		0.08030
Wolf	NA	NA	NA	NA	

Figure 1

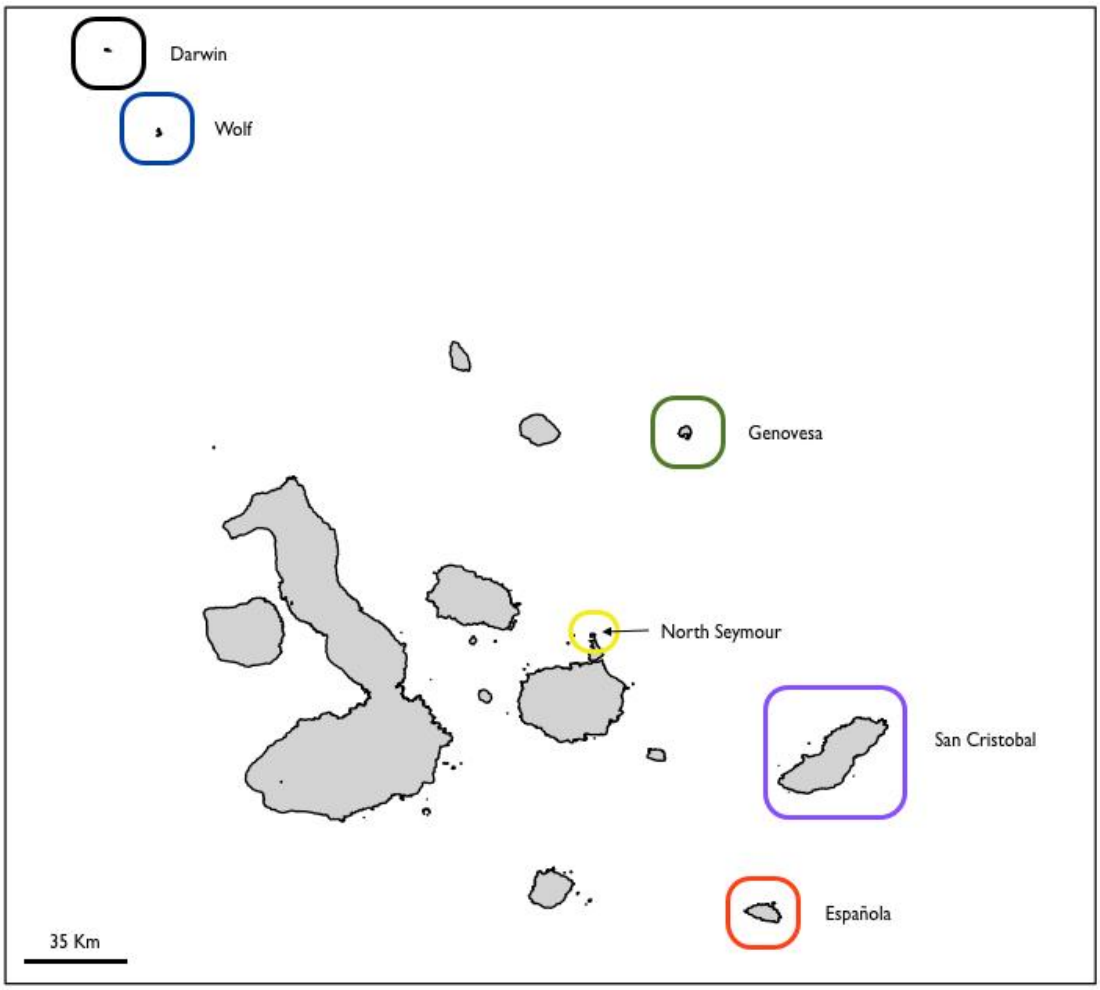
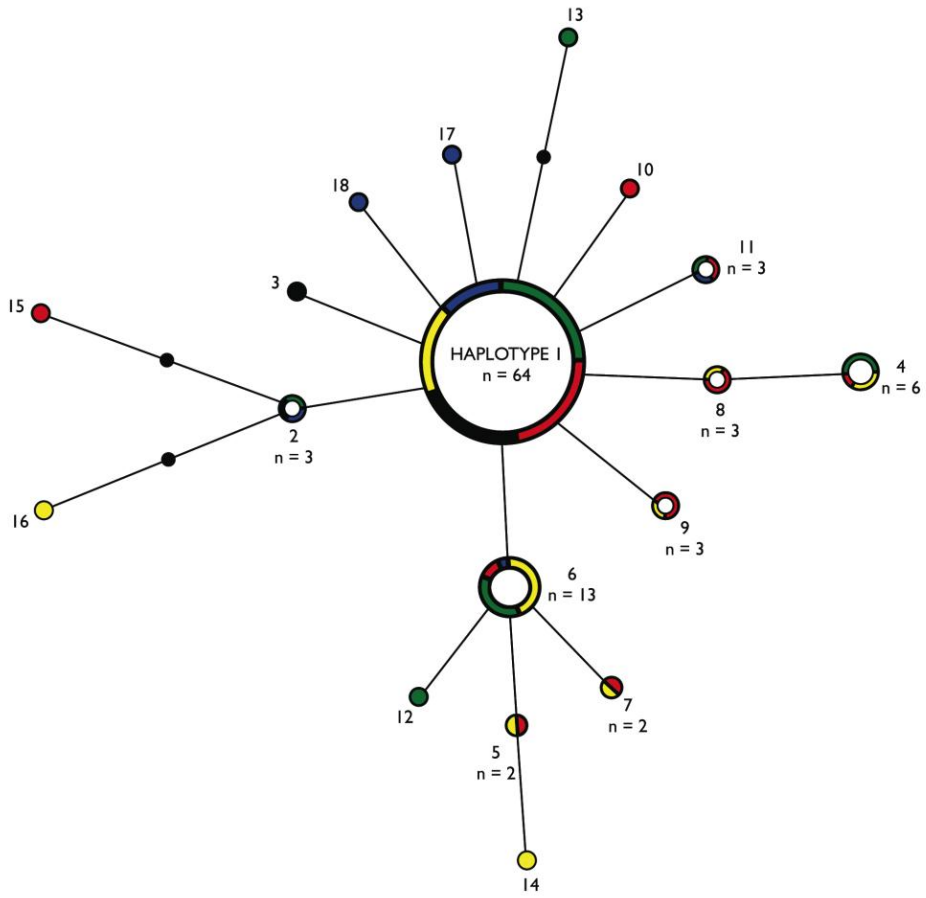

Darwin
Wolf
Genovesa
North Seymour
San Cristobal
Española
35 Km

Figure 2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n = 2
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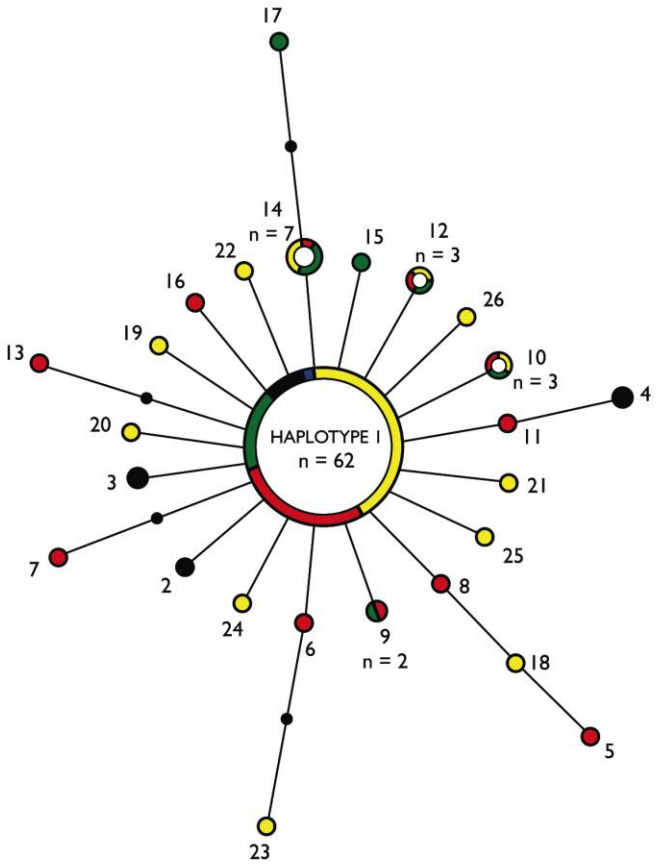
n = 2
14

Figure 3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HAPLOTYPE I
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25
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24
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n = 2
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18
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23

Figure 4

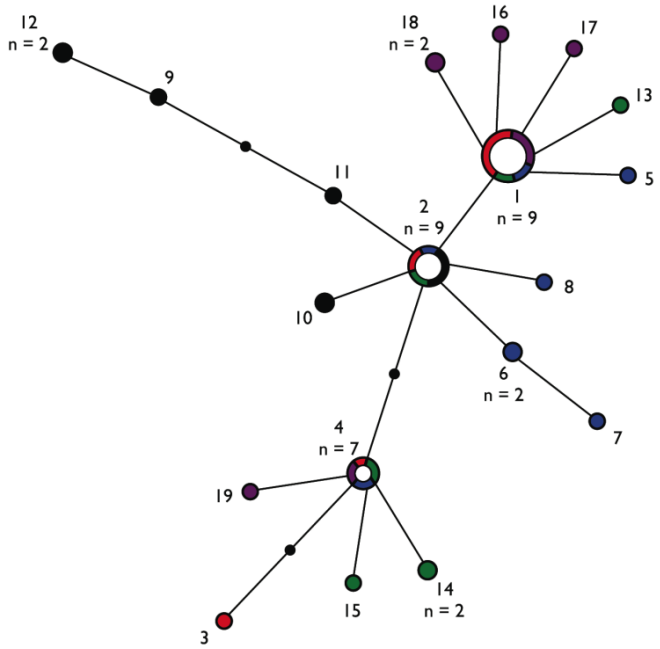
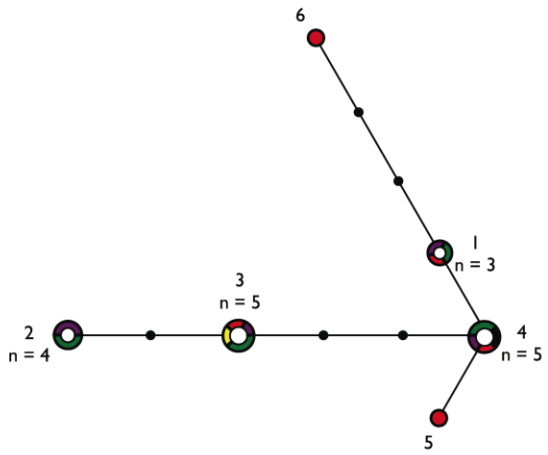


Figure 5



Chapter IX: Infection with *Haemoproteus iwa* affects vector movement in a Hippoboscid fly – Frigatebird system

Levin, I.I. and P.G. Parker, unpublished

Abstract: Studying haemosporidian parasites in their arthropod hosts in natural settings has proved challenging, especially in systems where the arthropod host is free-living. Here we explore the effects of a haemosporidian parasite, *Haemoproteus iwa*, on a Hippoboscid fly vector, *Olfersia spinifera*. *Olfersia spinifera* is an obligate ectoparasite of the Great Frigatebird, *Fregata minor*, living exclusively among bird feathers for all of its adult life. There is considerable evidence from mosquito – *Plasmodium* research that haemosporidian parasites can negatively impact their arthropod vectors. This study examines the movements of *O. spinifera* between Great Frigatebird hosts. Movement, or host-switching, is inferred by analyzing host (frigatebird) microsatellite markers run on DNA amplified from the vector. Using the most variable microsatellite markers, we are able to identify host genotypes in bloodmeals that do not match the host from which the fly was collected. We analyzed fly bloodmeal – host genotype mismatch using a logistic regression model, and the best-fit model included the *H. iwa* infection status of the fly and the bird host sex. Uninfected flies are more likely than infected flies to have a bird genotype in their blood meal that was different from that of their current bird host and flies collected from females were more likely than those collected from males to have a bird genotype in their blood meal that was different from that of their current host. Reduced movement of infected flies suggests that there may be a cost of parasitism for the fly. Parasite virulence reducing vector movement has been shown theoretically to be evolutionarily stable if that virulence contributes to a higher

success of infection (e.g., higher sporozoite production in the vector leading ultimately to an increased chance of infecting another vertebrate host). The effect of host sex on the probability of fly blood meal – host genotype mismatch could be driven by differences in *H. iwa* prevalence in male and female bird hosts and the sex of bird hosts available to moving flies. Males have a higher prevalence of *H. iwa* infection than females and breeding females spend proportionally more time in the colony as potential recipients of host-switching flies.

Introduction

Arthropod-vectored diseases are among the most damaging pathogens or parasites affecting human and wildlife populations. Historically, we have attempted to manage these diseases by focusing our control efforts on the vector, or alternatively attempting to enhance host resistance (Elliot et al., 2003). These approaches inevitably have evolutionary consequences for vectors and hosts, and there is growing interest in understanding evolutionary forces and responses in these systems (e.g., Cohuet et al., 2009). In many cases, the invertebrate vector is a far more elusive target of study than the vertebrate host, and laboratory experiments in model systems are often only remotely similar to natural host-parasite or host-pathogen interactions (Tripet et al., 2008). This presents challenges to studying host-parasite or host-pathogen interactions in their ecological and evolutionary contexts. Here we present a study of natural populations of a vertebrate host, the Great Frigatebird (*Fregata minor*), an invertebrate vector and obligate ectoparasite, the Hippoboscid fly *Olfersia spinifera*, and the haemosporidian parasite, *Haemoproteus iwa*. One of the features that make this system so tractable is the close association between vector and

vertebrate host; *O. spinifera* have fully functional wings but live exclusively among bird feathers for all life stages except the late-instar larval and pupal stages. Therefore, our ability to understand the movement of flies between bird hosts and the subsequent transmission of the haemosporidian parasite is more straightforward than in free-living vector systems (e.g., *Plasmodium* – mosquito – vertebrate host).

Haemoproteus iwa is a protozoan parasite that is found infecting frigatebirds throughout their tropical distribution (Levin et al. 2011). Based on *H. iwa* DNA amplification from *O. spinifera* thorax tissue (site of sporogony, the last developmental stage in the invertebrate), we have strong evidence supporting *O. spinifera* as the vector (Levin et al., 2011). The fitness consequences of an *H. iwa* infection for a bird host are not well understood apart from evidence of immune- or stress-response as indicated in blood smear differentials (Padilla et al., 2006) and correlative evidence showing an association between infection with *H. iwa*, elevated testosterone and a poorer quality sexual ornament important for mate attraction (Madsen et al., 2007). The impact of *H. iwa* on the Hippoboscid fly vector is even less well understood. It is not surprising that we lack information about the impacts on the vector; after nearly a century of study, the impacts of *Plasmodium* spp. parasites that cause malaria in humans on their mosquito vectors are unresolved (Ferguson and Read 2002). It has generally been predicted that, along with the potential for higher virulence than in non-vector-borne parasites, vector-borne parasites will be less virulent to the arthropod hosts than to the vertebrate hosts (Ewald 1994). Identifying the effects of these parasites on their arthropod hosts is pivotal in advancing understanding of the biology of human malaria and for

disentangling population-level processes occurring between parasites, vertebrate hosts and arthropod vectors.

The most well-studied haemosporidian parasite-vector system is *Plasmodium* spp. parasites and *Anopheles* spp. mosquitoes that cause millions of humans to become sick with malaria. There are several mechanisms by which *Plasmodium* parasites can damage the mosquito vector. First, passage of parasites through insect epithelia can cause physical damage and increase the susceptibility to bacterial infection (Hurd and Carter, 2004). In addition, there is evidence of physiological disruption in levels of mosquito digestive enzymes (Jahan et al. 1999) and resource depletion in the form of lower concentrations of amino acids (Beier 1998) and higher glucose usage (Hurd et al., 1995). Finally, there is evidence that mounting an immune response is costly to the mosquito (Tripet et al., 2008) and that some behavioral changes induced by infection, namely increased feeding and probing time, can result in increased risk of detection and consequently death of infected vectors (Ferguson and Read 2002).

This study examines the movements of *O. spinfera* between Great Frigatebird hosts. Movement, or host-switching, is inferred by analyzing host (frigatebird) microsatellite markers run on DNA amplified from the vector. Using the most variable microsatellite markers, we are able to identify host genotypes in bloodmeals that do not match the host from which the fly was collected. These mismatched host and vector-bloodmeal genotypes are then analyzed in a predictive model incorporating host biological and spatial information and host and vector infection status. We predicted that: (1) if there is an impact of the parasite on the vector we

would expect infected flies to move less, assuming movement is energetically costly to the vector; and (2) that host-switching by flies would be more likely in areas of high host density.

Materials and methods

Field sampling

Great Frigatebirds (*Fregata minor*) were sampled from five breeding colonies on different islands in the Galapagos archipelago, Ecuador (Darwin, Española, Genovesa, North Seymour, Wolf) in June and/or July of 2007, 2008 and 2010. Breeding adults were captured by hand at or near the nest. A blood sample was collected from the brachial vein and stored at ambient temperature in lysis buffer until DNA extraction. Hippoboscid flies (*O. spinifera*) were collected directly from the birds while sampling and stored in 95% ethanol at ambient temperature in the field and later at -20 C° in the laboratory until DNA extraction. A bird's sex was determined based on obvious sexually dimorphic plumage characteristics. Spatial data collected from each sampled bird included: distance from its nest to the nearest nest, number of nests within 10 meters, and the number of neighboring nests in 10 meters that were occupied by conspecifics. Bird-fly pairs (n=59) used in this study were selected prior to fly blood meal analysis using the following criteria: even sampling of the infected birds and infected flies, complete spatial information (unless the host was breeding, we did not collect spatial information), and pairs that were sampled from different islands. Because sampled bird hosts were breeding individuals, roughly half were of each sex.

Frigatebird DNA extraction and microsatellite amplification

DNA extraction, PCR techniques used to amplify *H. iwa* parasite DNA and sequencing follows Levin et al. (2011). Eight microsatellite markers (Fmin1, Fmin2, Fmin4, Fmin6, Fmin8, Fmin10, Fmin11, Fmin18) described in Dearborn et al. (2008) were used to characterize host genotype. With the exception of Fmin 6, Fmin8 and Fmin10, where the forward primer was fluorescently labeled, one primer in each of the remaining sets (typically the shorter one) had a 5' CAG tag applied (Glenn and Schable, 2005). We added a "pigtail" (GTTT) to the 5' end of the primer lacking the CAG tag to facilitate the addition of adenosine by the taq polymerase (Brownstein et al., 1996). Ten microliter PCR reactions were run using Bioline Red taq polymerase and accompanying reagents (Bioline, Tauton, MA). Microsatellites were amplified separately and then combined in two multiplex reactions with a size standard, GS500(-250)LIZ (Applied Biosystems (ABI), Life Technologies, Carlsbad, CA), to be read by the ABI 3100 Genetic Analyzer at the University of Missouri – St. Louis. Genemapper v.4.01 (Applied Biosystems, Life Technologies, Carlsbad, CA) software was used to analyze the fragment analysis results. All individual genotypes were manually scored, 10% of the total samples were repeated across all loci, and roughly one-third of all homozygotes were re-run to ensure we were not incorrectly assigning genotypes due to allelic dropout.

Fly DNA extraction and microsatellite amplification

In the laboratory, thoraxes of hippoboscid flies were separated from heads and abdomens. A Qiagen DNEasy Blood and Tissue DNA extraction kit (Qiagen, USA) was used to individually extract the DNA from each fly thorax. The standard

protocol was followed, but DNA was eluted in half as much buffer due to assumed low concentrations of any parasite or host DNA. Protocols for PCR amplification and sequencing were as described in Levin et al. (2011). To ensure that the positive PCR results from insects were DNA from sporozoites and not from undigested parasite-infected blood cells that might have persisted in the vector midgut as remnants of a blood meal, thoraxes of all flies were tested for bird mitochondrial *cyt b* gene with primers and protocols used in Ngo and Kramer (2003). We interpreted the PCR-positive flies as carrying infective sporozoites only when they did not also test positive for bird DNA in the thorax extracts. Frigatebird mitochondrial DNA was used as a positive control to identify and compare bird DNA amplified from insect thoraxes. In cases where no host DNA would amplify from thorax tissue, DNA was extracted from abdomens following the standard protocol recommended for the Qiagen DNEasy kit referenced above. We extracted DNA from fly abdomens in ten individuals that had host DNA in the thorax extraction to confirm we did not get conflicting results from the two different tissues. Four of the frigatebird microsatellite markers described above (Fmin2, Fmin6, Fmin10, Fmin18) were run on either fly thorax or abdomen extracts using the same protocols described above. These four primers were found to be most polymorphic in the bird host and therefore most informative for determining if the bloodmeal in the fly matched the genotype of the host from which the fly was collected. A subset of the flies were analyzed at six or all eight microsatellites to confirm that using the four most polymorphic markers was sufficient for identifying mismatched genotypes. Fly bloodmeal genotypes were scored without knowledge of the bird host genotype and the data were coded as

'mismatch' if at least one locus had different alleles in bloodmeal vs. host. If three or more alleles were found at any locus or loci (as was the case for some flies that had evidence of recently biting more than one host), we coded a mismatch, even if there was a match for the host genotype among the 2+ bird genotypes in the fly.

Logistic regression analysis

Logistic regressions were run using the package *glmulti* (Calcagno and de Mazancourt 2010) implemented in R v.2.14. An exhaustive search was run on the seven parameters we postulated could affect movement of vectors between individual hosts: island, infection status of the vector, infection status of the bird host, bird host sex, distance to the nearest nest, the number of nests within ten meters, and the proportion of nests within ten meters that were conspecific. One additional parameter, fly tissue, was included in the exhaustive search to confirm that there was no influence of using either thorax or abdomen tissue for extracting and amplifying bird DNA. An additional model was tested using the parameters listed above and the interaction between bird host sex and fly infection status. We used the Akaike Information Criterion (AICc) (Akaike 1974) for model selection and Wald tests to evaluate the significance of the parameters in the best model. To access the goodness-of-fit of the best model we ran a modified Hosmer-Lemeshow test in R using the package LDdiag (http://cran.rproject.org/web/packages/LDdiag /index.html).

Results

Of the 59 bird host-fly vector pairs analyzed, 28 of the host birds were female and 31 were male. Samples per island ranged from two host-vector pairs from the

island of Wolf to 21 pairs from North Seymour. Twenty-four of the 59 flies (41%) were infected with *H. iwa*, while prevalence in the frigatebird hosts was 33/59 (56%). In accordance with a larger study of *H. iwa* prevalence in Great Frigatebirds (Levin and Parker, in review), male frigatebirds were more heavily parasitized by *H. iwa* than females (males: 21/31 infected, females: 12/28 infected). Thirteen of the 24 infected flies were on infected males, while only two infected flies were on infected females. Thirty-seven of the fly vectors had bird microsatellite genotypes that did not match the host they were collected from. The best logistic regression model (determined by AICc values and residual deviances) included the infection status of the fly and the bird host sex (Table 1). Uninfected flies are more likely than infected flies to have a bird genotype in their blood meal that was different from that of their current bird host and flies collected from females were more likely than those collected from males to have a bird genotype in their blood meal that was different from that of their current host (Figure 1). Infected flies on female bird hosts had similar probabilities of genotypic mismatch as uninfected flies on male bird hosts (Figure 2). A modified Hosmer-Lemershow test showed no evidence for a lack of fit with this model ($p = 0.57$). A Wald's chi-square test indicated that the z-scores for both fly infection status and host sex coefficients were significant (Table 1) and that this logistic regression model including both fly infection status and bird host sex demonstrated a better fit to the data based on significant improvement over the null (intercept-only) model. The model search that also included the interaction between bird host sex and fly infection status produced the same best model as before,

including only bird host sex and fly infection status. The best model did not include any of the measured spatial parameters.

Discussion

Mismatches between vector bloodmeal genotype and bird host genotype were relatively high (37/59 or 62.7% mismatch). Previously, our only method of detecting potential host-switches was the occurrence of an infected fly on an uninfected bird (13/105 cases), which we acknowledge as an estimate of the lower bound of fly movement (Levin and Parker, in review). This approach using polymorphic, bird-specific, genetic markers is far more precise and provides more information about the recent movement of this vector. In one case, we were able to identify at least three bird genotypes in one fly. If fly movements between hosts are this frequent, it begs the question: why are some birds not infected with *H. iwa*? We argue that this could be a function of reduced movement by infected flies.

Our results reveal a striking pattern in recent vector movement: infected flies were more likely to have bloodmeals that matched the genotype of their current host than uninfected flies. Uninfected flies were more likely to have recently been on another bird host, indicating that they are more mobile. This suggests that there may be a cost of parasitism for the fly. From the parasite's perspective, an infected vector that is less likely to move is problematic; however, we do document cases of recent movement of infected flies, despite being less likely. It is possible that the benefits to the parasite from the processes that result in reduced vector movement (e.g., replication of the parasite in vector tissue causing tissue damage and resource depletion) outweigh the cost of reduced connectivity between bird host individuals.

In other words, selection may be acting to increase the virulence of the parasite in the vector if that virulence translates to a higher chance of successful infection of another vertebrate host. This is opposite than the usual prediction of selective advantage in vectors less affected by infection (Cohuet et al. 2009) although whether these predictions of lower virulence to vectors have any empirical basis has been questioned (Elliot et al., 2003).

An Evolutionarily Stable Strategy (ESS) model based on predator-prey interactions with the inclusion of a parasite demonstrate that there is actually a rather narrow set of conditions under which we would expect lower virulence in the more mobile host (vector) (Elliot et al., 2003). This leads the Elliot and coauthors to call into question the biases in the diseases that have been studied or how they have been studied. Their model predicts non-zero virulence in the vector and they state that "parasite virulence may reduce the mobility of one of the hosts, generating positive feedback as this in turn selects for higher virulence towards this host" (Elliot et al., 2003). This fits our observations, where we see parasite transmission persisting in a system where the vectors' movements are affected by the parasitic infection. Parasite virulence reducing vector movement is evolutionarily stable if that virulence contributes to a higher success of infection (e.g., higher sporozoite production in the vector leading ultimately to an increased chance of infecting another vertebrate host).

Studies of *Anopheles* mosquitoes focus mainly on the effects of *Plasmodium* on fecundity and survival, since both, especially survival, are expected to have large impacts on *Plasmodium* transmission. Additionally, because mosquitoes are free-living vectors, it is hard to compare effects of parasitism on mosquito vector

movement to that of our obligate ectoparasite. It has been established that *Plasmodium*-infected mosquitoes have a higher biting rate, presumably due to the high number of parasites in the vector that disturb the efficacy of blood feeding (Rossignol et al. 1984; Wekesa, et al. 1992). Infected mosquitoes were found to have less of a particular platelet inhibitor than uninfected mosquitoes, causing them to spend more time feeding (Simonetti, 1996). If similar mechanisms are at work in our *Haemoproteus*-fly-bird system, we might predict that an infected fly will be reluctant to leave a host if it must feed at a higher rate. There is little information on the feeding rate of Hippoboscid flies, other than in *Crataerina pallida*, the obligate parasite of Common Swifts (*Apus apus*) that feed once every five days (Walker and Rotherham 2010). However, there are no data available for whether that changes if the fly is infected with *Haemoproteus* parasites.

The other clear pattern we observed is the effect of bird host sex on the probability that the fly bloodmeal genotype matches that of its bird host. This was a surprising result that is potentially difficult to explain. This could be driven by differences in *H. iwa* prevalence in male and female bird hosts and the sex of bird hosts available to moving flies. If a newly emerged adult fly (uninfected with *H. iwa* as there is no evidence suggesting vertical transmission of haemosporidian parasites) lands on a male frigatebird, it is more likely to become infected with *H. iwa* as males frigatebirds in this sample had a prevalence of 67.7% whereas females were only 42.8% infected. Because we have evidence that the infection status of the fly contributes to the probability of movement, a fly landing on a male frigatebird has a higher probability of getting infected and therefore remaining on that host than if it

had landed on a female frigatebird. This could explain why infected flies on male frigatebirds had the lowest probability of bloodmeal-host genotypic mismatch.

But why are female frigatebirds more frequently the recipients of a host-switching fly? If a newly emerged adult fly finds a female bird host, it has a lower probability of becoming infected with *H. iwa*, so it may not have its further movements impaired. However, if flies are moving between multiple birds as it seems, and not just host-seeking once upon emergence from the pupa, this logic becomes more difficult, particularly because we do not know the sex of the previous host from which the fly came. However, we do know that during the breeding season (the time of sampling), female frigatebirds bear proportionally more of the reproductive effort as measured by time spent incubating the egg (Dearborn et al., 2001). Great Frigatebirds on Tern Island in Hawaii spent, on average, 10 more of 57 days incubating the egg than males, and there is strong evidence that, when not incubating, the other member of the pair is not present in the colony (Dearborn et al., 2001). This translates to breeding females spending roughly 18% more time in the colony than breeding males and therefore the more likely recipients of flies moving between individuals.

Using host-specific microsatellite markers on vector bloodmeals has proved to be a novel and exciting way to analyze recent vector movement, uncovering exciting patterns that fit predictions of ESS models for virulence evolution. This approach provides a wealth of information in our system where the vector is a host-specific, obligate parasite. Furthermore, it highlights *Haemoproteus* parasites and their Hippoboscid fly and bird hosts as an ideal system to study host-parasite interactions,

particularly for investigating the impacts of the haemosporidian parasite on the vector. Decades of laboratory research on mosquito-*Plasmodium* model systems have emphasized how specifically and intimately mosquito and parasite traits coevolve, and how context dependent the outcomes can be (Tripet et al. 2008). Together, these highlight the need to work with these parasite-vector-host systems in natural settings.

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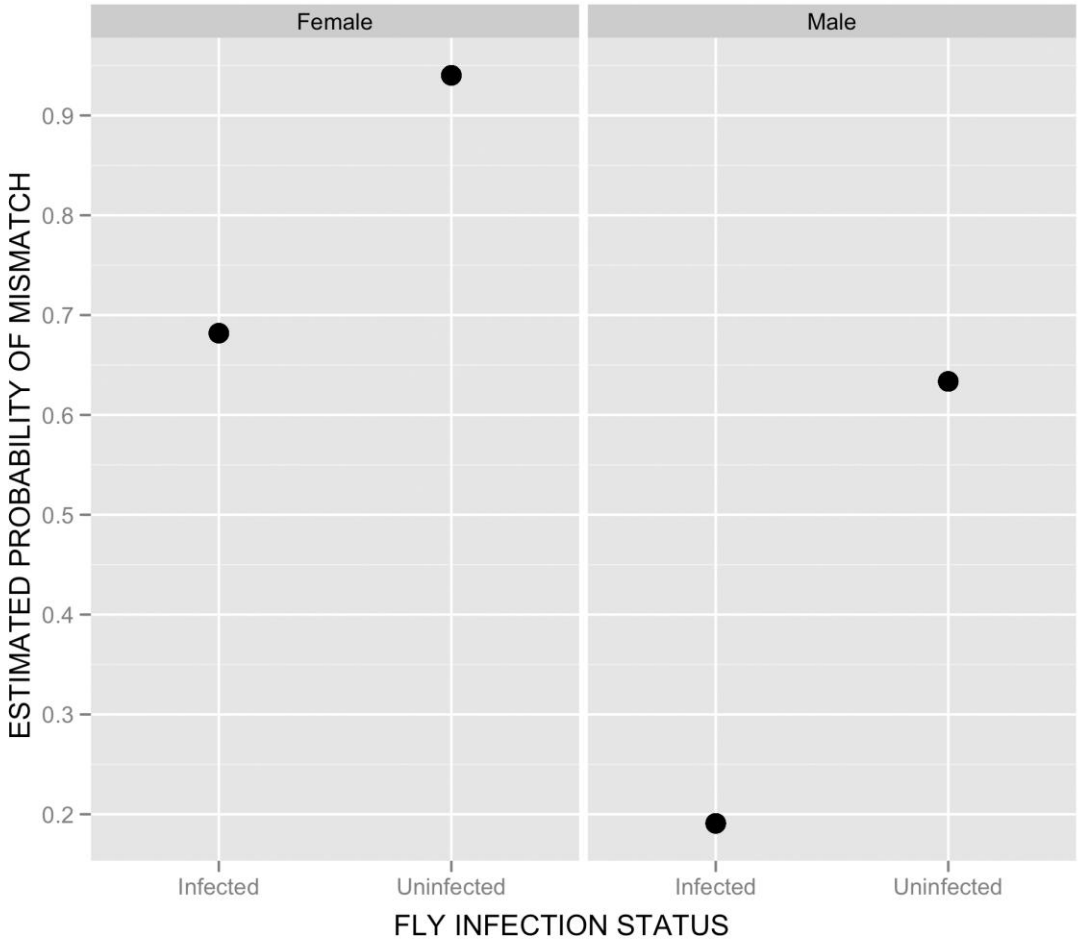
Figure legends

Figure 1: Estimated probability of mismatch between Hippoboscid fly (*Olfersia spinifera*) bloodmeal microsatellite genotype and Great Frigatebird (*Fregata minor*) microsatellite genotype for Hippoboscid flies infected with *Haemoproteus iwa* and free of infection split by host sex.

Table 1: Best fit logistic regression model as determined by the program *glmulti* run in R v. 2.14. Additional parameters used in the model search: Island, infection status of the vector, infection status of the bird host, bird host sex, distance to the nearest nest, the number of nests within ten meters, the proportion of nests within ten meters that were conspecific, and fly tissue used to amplify microsatellites.

Outcome: fly (*Olfersia spinifera*) blood meal matches/mismatches bird host (*Fregata minor*) genotype.					
Predictor	β (coefficients)	SE β	Wald's z-value	*df*	*p*
Fly infection status	1.9919	0.6872	2.899	56	0.00375
Host Sex	- 2.2068	0.7275	-3.033	56	0.00242
Null model	0.7625	0.6246	1.221	58	0.22219

Figure 1:


Female
Male
ESTIMATED PROBABILITY OF MISMATCH
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
Infected
Uninfected
Infected
Uninfected
FLY INFECTION STATUS