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# Haemosporidian Parasites and Host Immune Function of Galapagos Avifauna

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## Haemosporidian Parasites and Host Immune Function of Galapagos Avifauna

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A Dissertation Submitted to The Graduate School at the University of Missouri-St. Louis  
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2018

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## Table of Contents

Abstract.....	3
Dedication.....	4
Acknowledgements .....	5

### Chapters

<p><b>1. From Galapagos doves to passerines: spillover of <i>Haemoproteus multipigmentatus</i></b>.....</p> <p style="margin-left: 20px;">Jaramillo, M., Rohrer, S., &amp; Parker, P. G. (2017). From Galapagos doves to passerines: Spillover of <i>Haemoproteus multipigmentatus</i>. <i>International Journal for Parasitology: Parasites and Wildlife</i>, 6(3), 155-161.</p>	6
<p><b>2. No evidence for a role in avian malaria transmission by introduced birds in the Galapagos Islands</b>.....</p>	28
<p><b>3. Avian blood parasites (Haemosporida) in Galapagos: prevalence estimates that account for imperfect detection of the PCR test</b> .....</p>	52
<p><b>4. Exploring the relationship between immune function and birds' time since arrival to the Galapagos Islands</b>.....</p>	76

## ABSTRACT

The large number of emergent infectious diseases witnessed in the past few decades has increased interest in the ecology and distribution of potentially threatening pathogens worldwide. Island species are often considered more vulnerable to parasites due to their impoverished parasite communities, long isolation from disease and low genetic diversity. Avian surveys done by our group on the Galapagos Islands have found various pathogens infecting their endemic avifauna, including haemosporidian parasites of the genera *Plasmodium* and *Haemoproteus*. My research seeks to understand the relationships between two haemosporidian parasites (blood parasites) and their multiple bird hosts in Galapagos and to explore the immune system of insular birds. A three island survey was implemented along an altitudinal gradient between June 2013 and July 2015, to collect blood and plasma samples from 25 species of endemic and introduced birds. We explored patterns of *Haemoproteus multipigmentatus* infection in passeriform birds that provided evidence of parasite spillover events from Galapagos doves to passerines. We investigated the possibility that introduced birds in the archipelago were reservoir hosts for *Plasmodium* sp. But, contrary to our expectations, we found no evidence to suggest introduced birds are implicated in haemosporidian transmission or maintenance. We used a site-occupancy approach to obtain informed and more precise estimates of prevalence for both parasites and the ecological factors influencing variation, to improve assessments of disease risk for the endemic avifauna. And lastly, we investigated the relationship between a species' time of arrival to the archipelago and strength of the immune response. We found no general trend, among six indexes of immune response, to indicate that species that arrived to the islands earlier have a weaker immune system function than more recent arrivals. Collectively, our research demonstrates the importance of community wide surveys to identify or dismiss possible agents and factors of disease, to understand host-parasite dynamics and to better assess the disease risks faced by wildlife.

## DEDICATION

*A mis papás, por el apoyo y la motivación que me brindan y que siempre estará ahí.*

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**1. From Galapagos doves to passerines: spillover of *Haemoproteus*  
*multipigmentatus***

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## Abstract

*Haemoproteus (Haemoproteus) multipigmentatus*, a haemosporidian parasite thought to be specific to columbiform birds, was detected in passeriform birds on Santiago Island in the Galapagos archipelago. We surveyed birds along an altitudinal gradient on the islands of Santa Cruz, Isabela and Santiago between June 2013 and July 2015. Molecular screening of 2,254 individuals from 25 species of endemic and introduced birds revealed clusters of passerine birds positive for *H. multipigmentatus* on Santiago Island that coincide with captures of Galapagos doves at sampled sites. Of 507 individuals from 10 species of endemic passerines sampled on Santiago, 58 individuals from 6 species were found positive (11% prevalence). However, no gametocytes were found in the blood smears of positive passerines, suggesting that these species are not competent hosts for the parasite. All 31 doves captured were positive and gametocytes were found upon microscopic examination of all thin blood smears (averaging 357 gametocytes per 10,000 erythrocytes). These findings indicate parasite spillover from doves to passerines, but that passerines are possibly not competent hosts for further parasite transmission. The endemic Galapagos dove acts as a reservoir host for the introduced *H. multipigmentatus*, however the effect of this parasite on passerines has not been studied. We report on these findings because parasites can have large effects on individual host populations and on the ecology of a community, but may go undetected.

## Keywords

*Haemoproteus*, spillover, introduced parasite, reservoir host, Galapagos, Ecuador



## 1. Introduction

Pathogens that become established in new environments can substantially affect the ecology of host populations and the biodiversity of entire communities (van Riper et al., 1986; Dobson and Foufopoulos, 2001; Mackenzie et al., 2004). Pathogens can arrive in new areas through co-introduction with an exotic host species. Parasite establishment will then depend on the establishment of the exotic host species, or on the presence or simultaneous introduction of alternative hosts and suitable vectors (Lymbery et al., 2014). If infection with the parasite reaches high prevalence in a host, this reservoir host can influence disease dynamics in one or multiple host species through pathogen spillover (Daszak et al., 2000; Power and Mitchell, 2004).

Perhaps the most striking and documented example of how introduced parasites can affect isolated wild avian populations is that of Hawaii, where the introduction of avian pox and avian malaria (*Plasmodium relictum*) resulted in major extinctions of Hawaiian avifauna and restricted the distribution of those birds that survived infection (Warner, 1968; Van Riper et al., 1986). Parasites of the genus *Plasmodium*, as well as other avian haemosporidians of the genera *Haemoproteus* and *Leucocytozoon*, are globally widespread and transmitted by blood-sucking insects of the order Diptera. Haemosporidian infection varies from being relatively benign in some adapted birds (Bennett et al. 1993), to having positive (Zylberberg et al., 2015) and negative effects on host fitness (Nordling et al., 1998; Valkiūnas, 2004; Marzal et al., 2005; Valkiūnas, 2006; Møller and Nielsen, 2007; Atkinson, 2008), to severe pathology in non-adapted birds resulting in mortality in some cases (Atkinson et al., 1988; Cardona et al., 2002; Ferrell et al., 2007; Donovan et al., 2008; Olias et al., 2011; Cannell et al., 2013).

In the Galapagos Islands, several pathogens and parasitoids pose a risk to the avifauna of the archipelago (Fessl and Tebbich, 2002; Kleindorfer and Dudaniec, 2006, Santiago-Alarcon et al., 2008; O'Connor et al., 2010; Parker et al., 2011; Levin et al., 2013). Our research group made the first report of a *Plasmodium* spp. parasite in Galapagos penguins (*Spheniscus mendiculus*) (Levin et al., 2009). More recently, 4 lineages of avian *Plasmodium* have been described after screening numerous species of Galapagos birds (Levin et al., 2013). Other Haemosporidian parasites found on the islands include *Haemoproteus iwa* in frigatebirds (*Fregata minor*) (Levin et al., 2011), *Haemoproteus jenniae* in swallow-tailed gulls (*Creagrus furcatus*) (Levin et al., 2012), and *Haemoproteus multipigmentatus* in Galapagos doves (*Zenaida galapagoensis*) (Valkiūnas et al., 2010).

*Haemoproteus multipigmentatus* occurs with high prevalence in the Galapagos dove (Santiago-Alarcon et al., 2008). It appears to be widespread in the Neotropics and phylogenetic evidence suggests that the parasites found in Galapagos doves were likely introduced recently with domestic rock pigeons (*Columba livia*) brought to the islands (Santiago-Alarcon et al., 2010; Valkiūnas et al., 2010). Later re-screening, following Waldenström et al. (2004), of thirteen introduced rock pigeons used in that study revealed 6 individuals infected with *H. multipigmentatus*, reaffirming the suspicion that rock pigeons were implicated in the introduction of the parasite to the archipelago. Rock pigeons were completely eradicated from the Galapagos archipelago in 2007 (Phillips et al., 2012), but their blood parasites were already established in the Galapagos dove population and thus remained in the islands.

*H. multipigmentatus* is a parasite of the subgenus *Haemoproteus*, thought to be specific to columbiform birds (Valkiūnas et al., 2010). However, recurrent molecular

signals of *H. multipigmentatus* infection in endemic passerines of the archipelago show that the parasite infects other non-dove hosts as well. Sari et al. (2012) reported *H. multipigmentatus* infections detected by molecular screening of five Galapagos flycatchers (*Myiarchus magnirostris*) from Santa Cruz Island. Examination of thin blood smears of these individuals revealed no evidence of erythrocytic development of the parasite, indicating that Galapagos flycatchers were likely not competent hosts. Other instances of detection of infection with *H. multipigmentatus* have been documented by an ongoing large-scale avian disease survey that began in 2001 (Parker et al., 2006; Parker, 2009; 2016). However, these instances were too few and too scattered around the archipelago to determine their link to *H. multipigmentatus* prevalence.

Here, we present the results of our most recent surveys (2013-2015) of haemosporidian parasites in the Galapagos archipelago. We discuss how parasite spillover to passerine hosts might be shaped by the presence and abundance of an endemic reservoir host, the Galapagos dove. Furthermore, due to the specificity of *Haemoproteus* (*Haemoproteus*) to columbiform birds we expected Galapagos passerines to be non-competent hosts. However, even if the parasite did not reach the gametocyte stage in the blood stream of passerines, it might still affect host individuals if there is pre-erythrocytic development of the parasite in tissues (Olias et al., 2011; Cannell et al., 2013). Thus, we examined total leukocyte counts, polychromatophilic erythrocytes, and the heterophil to lymphocyte ratio (H/L-ratio) of infected individuals and uninfected individuals, as measures of immune activity, anemia, and chronic stress in reference to parasitism (Davis et al., 2008; Clark et al., 2009). Generally, the H/L-ratio is expected to increase in individuals responding to

disease and elevated leukocyte counts are characteristic of inflammatory processes in response to infection.

The simplicity of the Galapagos host-parasite system, in comparison to mainland ecosystems in which co-infections are common and many haemosporidian species and lineages are found, provides an excellent opportunity to study the prevalence of disease in avian hosts. Furthermore, we report on these findings because parasites can have a large effect on host populations and the ecology of a community, but may go undetected due to their cryptic nature. Parasite spillover is the first stage of host-switching, a common strategy in the evolution of avian haemosporidians (Bensch et al., 2000; Ricklefs et al., 2004; Galen and Witt, 2014; Ricklefs et al., 2014). However, the threat that these spillover events pose to non-competent hosts is often unknown.

## **2. Materials and methods**

### *2.1 Sample collection*

We sampled birds on an altitudinal gradient on Santa Cruz (6 sites at 4 different elevations), Isabela (8 sites at 3 different elevations), and Santiago Islands (3 sites at 2 different elevations), between June 2013 and July 2015 (Figure 1). We used 2.5 to 6-m tall mist nets to capture passerines and other land birds and a 12×15m drop net to capture cattle egrets. The number of birds captured divided by the sampling effort (nets x sampling hours) was used as a measure of relative abundance. After capture, morphological measurements were taken and birds were fitted with a plastic band (2013) or an aluminum band containing a unique number (2014-15). A small sample of blood, a volume proportional to less than 1% of their body weight, was taken by puncture of the brachial vein. One blood drop was used for each of two thin

blood smears; the smears were fixed in 100% methanol within one hour and stained with Giemsa within one month of collection. The remainder of the blood sample was centrifuged in the field for serum (for a concurrent study); red blood cells were separated and placed in lysis buffer (Longmire et al., 1988) for later genetic analysis.

## 2.2 PCR-based parasite screening

DNA extractions were performed using a standard phenol-chloroform extraction protocol (Sambrook et al., 1989), with a final dialysis step in 1M Tris pH 8, 5M NaCl, 0.5M EDTA, dH<sub>2</sub>O solution (40x TNE2). Extracted DNA was inspected with an Epoch spectrophotometer (BioTeK Instruments, Inc.) for adequate OD<sub>260</sub>/OD<sub>280</sub> values and DNA concentrations. For PCR-based molecular screening, a region of the parasite mitochondrial cytochrome b gene was amplified following the modified Waldenström et al. (2004) protocol in Levin et al. (2011). One microliter of stock DNA was used in the initial reaction and one microliter of amplicon from the initial reaction was used for the nested re-amplification reaction. Positive and negative controls were always used; the positive control was a consistently amplifying PCR-positive Galapagos penguin (*Spheniscus mendiculus*) and the negative control consisted of all PCR reagents without DNA. PCR-positives were sequenced to identify *Plasmodium spp.* and *Haemoproteus spp.* lineages. Parasite DNA amplified from PCR-positive individuals was purified and sequenced following Levin et al. (2011) on an ABI 3130 automated sequencer. DNA sequences were aligned and edited using Geneious version 10.1.3 (<http://www.geneious.com>, Kearse et al., 2012). Parasite lineages were identified through the Basic Local Alignment Search Tool (BLAST) in GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>) and the MalAvi database (<http://mbio-serv2.mbioekol.lu.se/Malavi/>; Bensch et al., 2009).

### 2.3 Microscopy

Blood smears of all infected passerines (n=58), doves (n=31) and a subset of uninfected passerines (n=14), were examined for presence of gametocytes at low magnification (x400) for five minutes followed by examination of 200 fields under high magnification (x1000) with an Olympus CX31 microscope. We performed leukocyte counts for individuals of *Geospiza fuliginosa* and *Geospiza fortis*, and for Galapagos doves, only in 2015, due to the suboptimal quality of our thin blood smears in other years. Leukocyte differentials, total leukocyte counts, and counts of thrombocytes and polychromatophilic erythrocytes in 10,000 erythrocytes were performed from images collected with a Leica ICC50 HD camera at high magnification (x1000). Erythrocyte counts were done through analysis of images with ImageJ, a java-based program that can recognize and count cell nuclei (Gering and Atkinson, 2004; Schneider et al., 2012). Heterophil to lymphocyte ratios (H/L-ratio) were compared between infected and non-infected groups by the Wilcoxon rank sum test in R. Total leukocyte counts, and number of leukocytes in  $10^4$  erythrocytes, were log transformed and compared with a two-sample t-test in R.

### 3. Results and Discussion

We screened 2,254 individuals from 25 species of endemic or introduced birds on the three islands. *Haemoproteus multipigmentatus* was found in passerines in all three sampling sites on Santiago Island and in only one medium ground finch (*Geospiza fortis*) on Santa Cruz Island (Figure 1). Galapagos doves were captured only on Santiago and all 31 doves sampled were found positive for *H. multipigmentatus*. Out of 507 individuals from 10 species of endemic passerines sampled on Santiago, 58 individuals from 6 species were found positive by PCR

(Table 1). Lineages MICRO01, ZEGAL05 and ZEGAL07 (Valkiūnas et al., 2010, Levin et al., 2011) were retrieved from doves and passerines. Even though doves have an archipelago-wide distribution and show a genetic population structure indicative of high levels of gene flow between islands (Santiago-Alarcon et al., 2008), during this survey they were not captured in any of the other survey sites on the islands of Santa Cruz or Isabela. This suggests that spillover of the parasite occurs only in sites where doves are present or sufficiently abundant to be detected by our capture technique.

On Santiago, mean passerine relative abundance was higher at the Lagoon locality, with 8.65 birds per net hour, than at Espumilla and Sullivan with 1.42 and 1.44 birds per net hour respectively (one-way anova,  $F_s=23.56$ ,  $p=0.002$ , Figure 2a). On average, the relative abundance of doves between sites was 0.22 doves per net hour at Sullivan, 0.07 doves per net hour at Espumilla and 0.03 doves per net hour at Lagoon (one-way anova,  $F_s= 2.1$ ,  $p= 0.27$ ). Mean prevalence of *H. multipigmentatus* in passerines was 21% at Sullivan, 14% at Espumilla and 10% at Lagoon (one-way anova,  $F_s=0.8$ ,  $p=0.5$ ). A slight, but not statistically significant, pattern is observed in which prevalence in passerines is higher at localities where more doves were captured (Figure 2a, b). A more appropriate study design to determine bird abundances would be needed to support this pattern and to determine whether dove abundance or *H. multipigmentatus* infection influences passerine abundance.

Gametocytes were found in thin blood smears of all Galapagos doves sampled (averaging  $357 \pm 307$  in 10,000 erythrocytes); however, no gametocytes or trophozoites were found in the blood smears of PCR-positive ( $n=58$ ) or PCR-negative ( $n=14$ ) passerines. This suggests that Galapagos endemic birds are possibly not competent hosts for *H. multipigmentatus* and our PCR method may be detecting

remnants of abortive development of the parasite that may be arrested at the tissue stages of the life cycle without being able to reach the erythrocytic stages of development. An alternative explanation is that we may be amplifying DNA from sporozoites injected by hippoboscid flies. We have not observed evidence of sporozoites or remnants of tissue meronts on the blood smears examined.

Previous research on *Plasmodium* spp. PCR-positive Galapagos birds presented similar findings to this in which the parasites are absent in erythrocytes (Levin et al., 2013). Furthermore, recent research of blood parasites in Andean passerines found hummingbirds are reservoirs of generalist *Haemoproteus* parasites, which probably spill over to other passerines; this study also found gametocytes only in hummingbirds (Moens et al., 2016). Another study of haemosporidian parasites in captive birds from the São Paulo Zoo, Brazil, also found several cases where blood stages were not detected by microscopic examination of PCR-positive bird smears (Chagas et al., 2017). It is becoming clear that this is a real issue in research of avian haemosporidians in wildlife, as parasite detection through molecular methods but not through microscopy suggests the possibility of abortive development in the tissue stage (Valkiūnas et al., 2014) that cannot be confirmed unless histological studies are performed (Dinholp et al., 2011; 2015; Ilgūnas et al., 2016; Palinauskas et al., 2016).

If the parasite is undergoing abortive development, there may be implications for the virulence of the parasites in these non-competent or non-adapted endemic passerines. Bird mortalities due to *Haemoproteus* spp. infection have been reported recently for captive exotic parrots in Europe (Olias et al., 2011) and wild Little penguins, *Eudyptula minor*, in Australia (Cannell et al., 2013). Myocardial, skeletal muscle, hepatic and splenic necrosis in these cases were associated with abortive



infections, where parasite development has not been completed and is still at the tissue stage, before the development of gametocytes or presence of parasites in blood (Valkiūnas, 2011). This suggests that *Haemoproteus* spp. infections might be lethal to some non-adapted hosts at early stages of parasite development (Valkiūnas, 2011) and warrants further research at the tissue stage for Galapagos hosts.

The results from our comparisons of total leukocyte counts and the heterophil/lymphocyte ratio (H/L-ratio) between PCR positive and negative individuals were opposite of what might be expected. The H/L-ratio was significantly lower for positive ( $0.13 \pm 0.06$ ,  $n=10$ ) than for negative *G. fuliginosa* ( $0.7 \pm 0.7$ ,  $n=6$ ,  $p=0.01$ ). For *G. fortis* the H/L-ratio was higher for positive individuals ( $0.74 \pm 0.54$ ,  $n=3$ ) than for negative ( $0.03 \pm 0.01$ ,  $n=3$ ,  $p=0.1$ ), although the difference was not significant due to small sample size. For *G. fuliginosa*, the total leukocyte count was significantly 59% lower for PCR-positive individuals than for negative individuals but for *G. fortis* the difference was not significant, even though it was 48% lower for positive individuals than for negative individuals (Table 2), likely due to small sample size. Again, contrary to what is expected in case of disease or stress for *G. fuliginosa*, we found significantly fewer monocytes and eosinophils in positive than in negative individuals, and for *G. fortis*, significantly fewer lymphocytes in positive than in negative individuals (Table 2). We must note that our capture method (mist netting) may only sample actively flying individuals; sick individuals usually have reduced activity or die rapidly during abortive development of tissue stages of the parasite (Yorinks and Atkinson, 2000; Valkiūnas and Iezhova, 2017). Thus, we might have sampled birds at very early stages of infection or that had already recovered, which may explain our immunological observations. Further research with larger sample sizes, alternative capture methodologies, histological examination of dead birds or

experimental infections would elucidate our understanding of the physiological responses of infected individuals.

In doves, we found a positive association between the number of gametocytes in 10,000 erythrocytes and the number of polychromatophilic erythrocytes ( $r^2=0.49$ ,  $p=0.001$ ). This may indicate stimulated erythrocyte production to overcome mature erythrocyte loss as intensity of infection increases. The demand on the host to produce more erythrocytes suggests a cost of infection to doves. In some columbiform birds, histopathological examination of dead doves' organs has revealed sufficient tissue damage to assume infection with *Haemoproteus columbae* as cause of mortality (Earle et al., 1993). On the other hand, doves presented a weak negative association between the number of gametocytes in 10,000 erythrocytes and the H/L-ratio ( $r^2=0.24$ ,  $p=0.03$ ), while the total number of white blood cells did not show a relationship with the number of gametocytes in 10,000 erythrocytes ( $r^2=-0.07$ ,  $p=0.911$ ). Thus, better measures of chronic stress or immune response would be needed to understand response to infection in Galapagos doves as well as in passerines.

Possible vectors of *H. multipigmentatus* include the hippoboscid flies, *Microlynchia galapagoensis* found in Galapagos Doves (Valkiūnas et al., 2010) and *Ornithoica vicina* found in Galapagos passerines (Deem et al., 2011; Sari et al., 2012). However, we were not able to sample hippoboscid flies from the captured birds; the flies are perhaps lost when the birds are captured in the nets. Thus, other capture and sampling methods may be necessary to determine the inter-species vector of *H. multipigmentatus*.

#### **4. Conclusion**

We found that clusters of *H. multipigmentatus*-positive passerines on the island of Santiago coincided with clusters of Galapagos dove captures. The co-occurrence of doves with infections in passerine birds, together with the 100% prevalence and the presence of gametocytes in Galapagos doves, suggest that the parasite is being transmitted from doves to passerines. The degree of transmission may depend on whether doves are present and how abundant they are at any given site. The absence of gametocytes in passerine blood smears indicates that they are possibly dead-end hosts; however, future research should aim at determining any effects of *H. multipigmentatus* on the survivorship and reproduction of these Galapagos endemic birds. Many parasites go unnoticed owing to their cryptic nature, but findings such as ours provide an opportunity for further research on how introduced parasites affect non-competent hosts in island communities.

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## Legends to figures

Figure 1. Map of the Galapagos Islands indicating sampling sites during our haemosporidian survey 2013-2015. Prevalence percentages are provided only for those sites where *Haemoproteus multipigmentatus* was found.

Figure 2. a. Mean prevalence of *Haemoproteus multipigmentatus* in passerines on sampling sites on Santiago. b. Mean relative abundance of doves and passerines on sampling sites on Santiago. Bars indicate standard error. (Sullivan was sampled only in 2014 and 2015)

## Tables (with their legends)

Table 1. Number of individual birds tested by PCR for *Haemoproteus* spp. by survey site on Santiago. Numbers in parentheses indicate the number of individuals infected with *Haemoproteus multipigmentatus*.

Species	Common name	Espumilla	Lagoon	Sullivan	Total
<i>Platyspiza crassirostris</i>	Vegetarian Finch	-	1	-	1
<i>Camarhynchus parvulus</i>	Small tree finch	7	11	-	18
<i>Camarhynchus psittacula</i>	Large tree finch	1 (1)	-	-	1 (1)
<i>Geospiza fortis</i>	Medium ground finch	21 (1)	103 (7)	2 (1)	126 (9)
<i>Geospiza fuliginosa</i>	Small ground finch	72 (12)	140 (16)	18 (4)	230 (32)
<i>Geospiza magnirostris</i>	Large ground finch	2 (1)	12	-	14 (1)
<i>Geospiza scandens</i>	Cactus finch	1	2	-	3
<i>Myiarchus magnirostris</i>	Galapagos flycatcher	6	22	-	28
<i>Mimus parvulus</i>	Galapagos mockingbird	1 (1)	24 (6)	-	25 (7)
<i>Setophaga petechia</i>	Yellow warbler	8 (1)	50 (7)	3	65 (8)
<i>Zenaida galapagoensis</i>	Galapagos dove	9 (9)	4 (4)	18 (18)	31 (31)
Totals		128 (26)	369 (40)	41 (23)	538 (89)

Table 2. Polychromatophilic erythrocytes and leukocyte counts for PCR positive and negative *G. fuliginosa* and *G. fortis*, based on cell counts over  $10^4$  non-polychromatophilic erythrocytes.

	<i>Geospiza fuliginosa</i>			<i>Geospiza fortis</i>		
	PCR- negative (n=11)	PCR- positive (n=10)	P	PCR- negative (n=3)	PCR- positive (n=3)	P
<b>PE</b>	464.3 (209.8)	449.0 (157.3)	0.98	399.9 (36.4)	495.6 (115.5)	0.251
<b>Heterophils</b>	11.7 (10.0)	3.9 (3.3)	0.09	2.0 (1.7)	17.7 (15.5)	0.13
<b>Lymphocytes</b>	23.1 (16.5)	18.9 (13.3)	0.65	64.7 (21.1)	15.3 (11.2)	<b>0.03</b>
<b>Monocytes</b>	2.8 (2.7)	0.8 (1.6)	<b>0.02</b>	2.3 (1.5)	1.7 (1.5)	0.57
<b>Eosinophils</b>	4.5 (3.7)	1.5 (1.6)	<b>0.03</b>	1.7 (1.5)	0 (0)	0.19
<b>Basophils</b>	0 (0)	0 (0)	NA	0 (0)	0 (0)	NA
<b>Total leukocyte</b>	42.0 (21.6)	25.0 (17.1)	<b>0.02</b>	70.5 (19.5)	34.5 (19.0)	0.13

Mean and standard deviation (in parenthesis) provided. P values from the comparison between positive and negative individuals' log (+1) transformed counts included. Significant values provided in bold.

## **2. No evidence for a role in avian malaria transmission by introduced birds in the Galapagos Islands**

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**Abstract**

Detection of a *Plasmodium* spp. parasite in Galapagos has raised interests about the pathogen's disease ecology on the archipelago. Recent work by our group found endemic birds are susceptible to infection but no evidence of the infective stages of the parasite has been observed (Levin et al. 2013). Repeated findings of a specific lineage in different species, different years, and on different islands suggest that there must be a reservoir species in the archipelago that will allow the parasite to become infective to a competent mosquito. We investigated the possibility that it may be one of the two exotic bird species with large breeding populations on the islands, the Cattle Egret (*Bubulcus ibis*) or the Smooth-billed Ani (*Crotophaga ani*). These birds' relatively recent arrival and their gregarious roosting and breeding habits made them candidates for reservoir hosts. In such case the eradication of avian malaria from the Galapagos archipelago would have been facilitated through eradication or control of these introduced birds. We screened blood and/or tissue samples from 194 egrets and 127 anis from the islands of Santa Cruz and Isabela. In addition, we screened samples of 483 Southern House Mosquitoes (*Culex quinquefasciatus*) and 3,273 Black Salt Marsh Mosquitoes (*Aedes taeniorhynchus*) surrounding introduced birds' feeding and/or roosting areas and 167 louse flies (Hippoboscidae) found on egrets. Contrary to our expectations, we found no evidence that these birds might have introduced or that they are reservoirs for the parasite. Our research contributes to the understanding of the disease dynamics of Haemosporidian parasites in the isolated avifauna of Galapagos and points to future directions.

**Keywords:** invasion, disease, introduced, *Plasmodium*, Galapagos avifauna

## Introduction

Invasive species are a leading cause of native biodiversity loss and ecosystem impacts worldwide (Pimentel 2011, Simberloff 2011). To become invasive, an alien species must surpass geographical barriers to arrive to a new area, overcome ecological barriers to survive and reproduce, and surmount dispersal barriers to spread (Blackburn et al. 2011). An invasive species may have direct or indirect effects on native species and habitats, and parasites may have an important role in the process of biological invasion (Lymbery et al. 2014). Likewise, introduced species can influence disease dynamics by amplifying (Kelly et al. 2009) or reducing (Poulin et al. 2011) transmission of enzootic diseases, or bringing new parasites into an area (Daszak et al. 2000, Dobson and Foufopoulos 2001).

Parasite transmission between invasive and native hosts may lead to the emergence of a new disease with significant consequences to native health and survival, which may be particularly true for species that have no evolutionary history with those parasites, such as island endemics. Avian haemosporidian parasites, which include avian malaria (*Plasmodium*) and other closely related haemosporidians (*Haemoproteus*, *Leucocytozoon* and *Fallisia*), are an example of a large group of globally widespread vector-borne parasites that have negative effects on fitness and survival of non-adapted hosts (Atkinson et al. 1988, Cardona et al. 2002, Valkiūnas 2005, Ferrell et al. 2007, Atkinson 2008, Donovan et al. 2008, Olias et al. 2011, Cannell et al. 2013). Introduced bird species, thus, may play an important role in haemosporidian parasite spread and maintenance in isolated archipelagos, due to their proven ability to surmount dispersal barriers and their likelihood of more recent exposure in their mainland site of origin.

In Hawaii, Van Riper et al. (1986) suggested that avian malaria was introduced through the numerous releases of exotic birds that preceded the large number of infected native birds found in the 1970's. Later, Beadell et al. (2006) showed that the *Plasmodium relictum* lineage found in Hawaii is common in Europe, suggesting an Old World origin for this parasite. The distribution of introduced birds at the time suggested they might have been reservoir hosts for the parasite. As native birds were restricted to the highlands, introduced birds increased in abundance with decreasing elevation and were particularly abundant in lowland mesic forest where both *P. relictum* and the mosquito vector were most abundant (van Riper et al. 1986). Furthermore, prevalence fluctuated with seasonality in native birds but tended to be more uniform throughout the year in introduced birds, which possibly facilitated the maintenance of *P. relictum* in the Hawaiian archipelago while dozens of endemic bird species were driven to extinction (van Riper et al. 1986). In New Zealand, six introduced avian malaria lineages have been discovered, all of which are common in Europe. As the majority of introduced avifauna in New Zealand are of European origin, this correlation points to a possible source of the pathogens (Ewen et al. 2012). Furthermore, a high prevalence of avian malaria infections in introduced blackbirds suggests they could be responsible for maintaining the disease in New Zealand (Tompkins and Gleeson 2006).

Our research group made the first report of a *Plasmodium* spp. parasite in the Galapagos archipelago, in Galapagos Penguins (*Spheniscus mendiculus*) (Levin et al. 2009). The lineage of *Plasmodium* found, is closely related to lineages that have caused high mortality in captive penguins. Since the initial discovery, our group has discovered additional lineages of *Plasmodium* in the archipelago after screening numerous species of Galapagos birds (Levin et al. 2013). One of the lineages present



in penguins (SPMEN03) was also found in Mangrove Warblers (*Setophaga petechia*) and one Medium Ground Finch (*Geospiza fortis*). This lineage has been recurrently found across years, in multiple species, and on multiple islands, as opposed to the other three lineages found in passerines that were detected briefly in one or two birds and disappeared shortly after.

While endemic Galapagos birds are susceptible to infection, the parasite does not seem to be well adapted to fully develop within these birds, since no evidence of the reproductive stages of the parasite has been found in the microscopy screening (Levin et al. 2013). However, repeated findings of SPMEN03 suggest that there must be a species in the archipelago that will allow the parasite to become infective to a competent mosquito by allowing the parasite to develop through to the final erythrocytic gametocyte stage. Here we investigate the possibility that it may be one of the two introduced species with large breeding populations in the islands, the Cattle Egret (*Bubulcus ibis*) or the Smooth-billed Ani (*Crotophaga ani*).

The Cattle Egret, first recorded in the 1960s, has had breeding populations in the islands since 1986 (Rosenberg et al. 1990). Although this species may have arrived by its own means, it is considered an introduced species by managers in the Galapagos in the sense that its establishment may have been facilitated by the existence of the islands' cattle industry. The Smooth-billed Ani was introduced by inhabitants of the islands in the 1960s under the popular assumption that they would feed on the ticks on cattle and therefore act as a biological control, which is suggested by the bird's common name in Spanish: "Garrapatero" which translates to "tick-eater" (Wiedenfeld 2006).

These two introduced birds are gregarious animals that roost and breed in colonies in abundances that may promote host-feeding by mosquitoes (Burkett-Cadena et al. 2011). For the egrets, these colonies are often close to bodies of water (i.e. lagoons, ocean) scattered through the inhabited islands of the archipelago (Wiedenfeld 2006). Positive associations found between haemosporidian parasite infection and proximity to water (Wood et al. 2007, Krams et al. 2010, Lachish et al. 2011, Krama et al. 2015) indicates these areas may provide suitable larval habitat for some vectors. These factors altogether suggest that these introduced birds may have been playing an elemental role in disease transmission.

If these birds were reservoirs of disease, we would expect to find measurable prevalence of the parasite and evidence of reproductive stages of the parasite in the host's blood that would indicate the parasite can complete its life cycle and become infective to a vector. The endemic Galapagos Dove (*Zenaida galapagoensis*) provides an example of a reservoir host of a haemosporidian parasite of the genus *Haemoproteus*. Galapagos Doves present a very high prevalence of infection (36 - 100%) and parasitemias of *Haemoproteus multipigmentatus* as high as 12.7% of red blood cells (Santiago-Alarcon et al. 2008). *H. multipigmentatus* is a parasite of the subgenus *Haemoproteus* that is thought to be specific to birds in the order Columbiformes (Valkiunas et al. 2010); however, in the presence of doves the parasite spills over to different species of Galapagos birds, implicating the doves as reservoir hosts (Jaramillo et al. 2017).

Vector competence and abundance are also likely to play a critical role in maintaining parasite transmission. In addition to identifying possible reservoirs for avian malaria, it would be important to identify the arthropod vector(s) involved in

this disease transmission cycle. There are currently three species of mosquitoes in Galapagos. *Aedes taeniorhynchus* or the Black Salt Marsh Mosquito lays its eggs in brackish water and is considered native to the archipelago given its natural arrival 200,000 years ago (Bataille et al. 2009). The Southern House Mosquito, *Culex quinquefasciatus*, a recent arrival in 1985 (Whiteman et al. 2005) is mainly found on human-inhabited islands such as Santa Cruz and Isabela (Asigau et al. 2017). This is due to its preference for egg-laying in fresh stagnant water, common near human habitations where it collects in old tires or containers. The Southern House Mosquito is known for its association with avian malaria transmission that led to the extinction of significant numbers of Hawaii's endemic avifauna in the 1800s (van Riper et al. 1986). *Aedes aegypti* or Yellow Fever Mosquito, the most recent arrival of the three species, was first recorded in the Galapagos in 2001 (Causton et al. 2006). Like the Southern House Mosquito, this species is found on human-inhabited islands such as Santa Cruz, Isabela and San Cristobal, however, it is thought to be highly anthropophilic (Causton et al. 2006) and thus is not likely to be involved in avian malaria transmission. If introduced birds were reservoirs for avian malaria, it is highly likely that infected mosquitoes would be found around their roosting or feeding sites, particularly those where there could be suitable mosquito larval habitat such as lagoons or stagnant water.

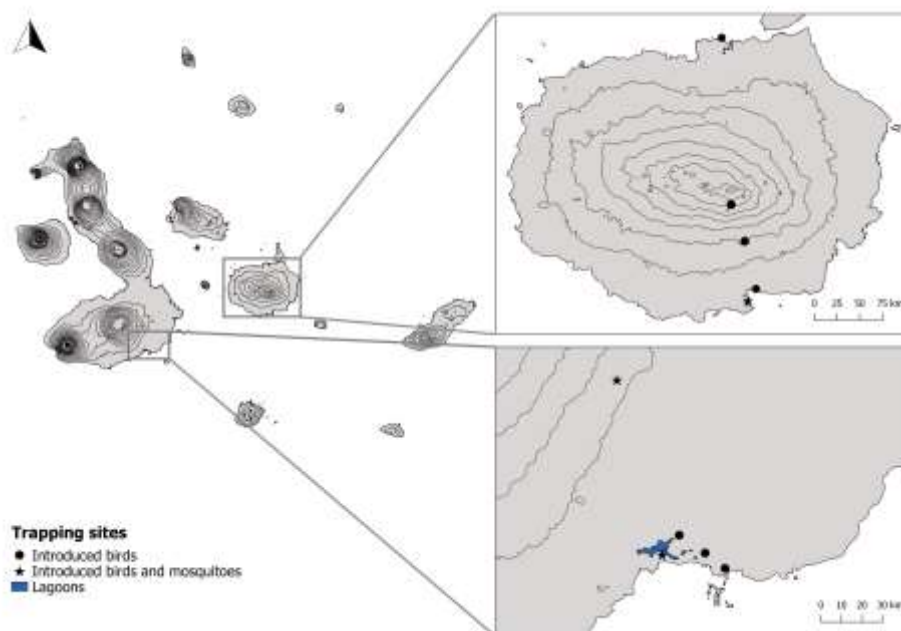
Here we assess *Plasmodium* spp. infection in introduced populations of the Cattle Egret and Smooth-billed Ani in the archipelago and in mosquitoes caught nearby to try to identify agents and/or hotspots of transmission, which could provide managers with a timely opportunity to prevent major losses of Galapagos avifauna. Investigating the disease dynamics of newly discovered malaria parasites in the Galapagos Islands provides an opportunity to understand the dynamics of introduced

diseases on islands and their association with introduced species. As biological invasions and emergent diseases are not necessarily independent from each other, understanding their synergistic effect on native species is a critical step in native species conservation.

## Methods

We captured introduced birds in Isabela and Santa Cruz islands (Fig. 1), two inhabited islands where the highest prevalence of *Plasmodium* spp. has been found (Levin et al. 2013). We sampled blood and liver tissue from dead Smooth-billed Anis shot by the Galapagos National Park during their efforts of population control. Live Smooth-billed Anis were caught with either 60mm mesh mist nets (playback was used on occasion) carefully placed in flying paths within ani territories or walk-in traps employed by Charles Darwin Research Station (CDRS) collaborators for a separate study.

**Figure 1.** Introduced bird and mosquito capture locations in Santa Cruz (top right) and Isabela (bottom right) islands.



To capture live Cattle Egrets, we explored several methods of capture which proved to be ineffective, including: walk-on traps (15m long plastic mesh with nylon noose knots), a 3x3x3m wood and plastic mesh funnel trap with organic trash and Cattle Egret decoys inside, 3m tall mist nets with decoys placed nearby, net guns and hand capture in roosting sites with noose knots tied to 7m long aluminum poles. We also fabricated three sets of 7m tall, 1" in diameter, aluminum stack poles that held two 2.6-3.2m high, 90-100mm mesh, 9-12m wide nets. The mist nets were set in the intertidal rock shore of mangrove trees at Punta Estrada, Santa Cruz to catch egrets coming down from the highlands to their roosting site at dusk. Also, Cattle Egret nestlings were hand caught at breeding sites in Santa Cruz and Isabela. These last two methods were effective but required large amounts of effort and time investment for a small number of captures. By far, the most successful method for catching egrets was a 9x12m, no. 18 green nylon, 2" stretched mesh bird drop net made for us by Debbie and Greg Faulkner at Innovative Net Systems (Milton, Louisiana). The drop net was held up with ropes, aluminum poles and aided with existing structures at the Santa Cruz and Isabela organic landfills (Fig. 2). The net was set up approximately an hour before dawn and the trigger was released when enough egrets were foraging on organic trash en route from coastal roosting sites to their daily foraging sites in the highlands of both islands.

**Figure 2.** Drop net at the organic landfill in Isabela (bottom). Egrets shown foraging under the drop net (top right), research assistants Jeisson Zamudio and Robert Jansen sampling a captured Cattle Egret (top left).



Morphological measurements were taken and birds were banded with an aluminum band containing a unique number to avoid re-sampling individuals. A small sample of blood, a volume proportional to less than 1% of individual body weight, was taken by puncture of the brachial vein. One blood drop was used for each of two thin blood smears; the rest of the blood was centrifuged for serum and red blood cells were separated and placed in lysis buffer (Longmire et al. 1988) for parasite screening. Blood smears were scanned for parasites at low magnification (x 400) for five minutes, followed by examination of 200 fields under high magnification (x

1000) with an Olympus CX31 microscope. Additionally, we opportunistically sampled louse flies (Hippoboscidae) when found on Cattle Egrets.

We sampled mosquitoes at the organic landfill on Isabela between 11<sup>th</sup> to 13<sup>th</sup> July, 2013 and 8<sup>th</sup> to 10<sup>th</sup> April, 2014 and at the breeding site for Cattle Egrets between 28<sup>th</sup> to the 30<sup>th</sup> of April 2014. Mosquitoes were sampled at the egrets breeding site at Punta Estrada in Santa Cruz between 15<sup>th</sup> to 17<sup>th</sup> and 17<sup>th</sup> to 19<sup>th</sup> July, 2014. We established two CDC light traps (Model 512 John Hock Company, Gainesville, FL) and two CDC gravid traps (Model 1712 John Hock Company, Gainesville, FL) and trapped mosquitoes for 3 - 4 consecutive days at each site, which coincided with mist-netting and bird capturing events. CDC light traps were baited with a CO<sub>2</sub> mixture containing 250g sugar, 35g yeast and 2.5 liters of water to lure host-seeking mosquitoes (Smallegange et al. 2010). Gravid traps were baited with hay-yeast-water infusion to attract ovipositing mosquitoes (Reiter 1986). Both trap types were set one hour before dusk and mosquitoes were collected one hour before dawn (~18:00 – 06:00 hours). Wild-caught mosquitoes were later immobilized with chloroform, sexed and identified to species level using morphological characters. Female mosquitoes were separated by head/thorax and abdomen regions using sterile techniques and pooled separately in 180 µL of Longmire's lysis buffer. Pool sizes varied from 1 – 10 mosquitoes and dissected regions were pooled by species, date of capture, trap type and site. Due to time constraints and to prevent mosquitoes from drying out in tropical conditions, whole mosquitoes not dissected were preserved in 95 percent ethanol in pools of 11 – 50, exported to the Parker laboratory at University of Missouri-Saint Louis, and then stored at -20 degrees Celsius.

We extracted bird DNA following a standard phenol-chloroform extraction protocol (Sambrook et al. 1989), with a final dialysis step in TNE<sub>2</sub> (1M Tris pH 8, 5M NaCl, 0.5M EDTA, dH<sub>2</sub>O solution). Genomic DNA from all hippoboscids and female mosquito tissues were extracted using a Machery Nagel NucleoSpin® Tissue Kit. For whole mosquitoes preserved in ethanol, we dissected individuals into head/thorax and abdomen regions on a fresh slide with a sterile scalpel prior to DNA extractions. Mosquito legs and wings were carefully removed prior to dissection. After each dissection, we sterilized scalpels by dipping them in 10 percent bleach, followed by distilled H<sub>2</sub>O, in 70 percent ethanol and finally in distilled H<sub>2</sub>O. Scalpels were further sterilized by applying heat for 3 seconds using a Bunsen burner. Head/thoraces and abdomen regions were put in separate microcentrifuge tubes and placed under a hood for 4 hours to remove any residual ethanol. Insects were homogenized using a heat-sealed pipette tip, placed in T1 buffer and DNA was extracted following the Machery Nagel NucleoSpin® Tissue Kit user manual guide (pp 13 - 15).

PCR screening was done following Waldenström et al. (2004), which searched for a region of the parasite mitochondrial cytochrome *b* gene. Positive and negative controls were always used; the negative control consisted of all PCR reagents without DNA, and the positive control was a consistently amplifying PCR *Plasmodium* positive Galapagos Penguin for initial screening. All avian samples were then diluted to DNA concentrations  $\leq 200$  ng/nl and screened an additional one to three times with a consistently amplifying *Haemoproteus*-positive Galapagos Dove. Insect samples were screened only once and all PCR-positives were cleaned and sent out for sequencing to Eurofins Genomics (12701 Plantside Drive | Louisville, Kentucky 40299). Identification of sequences was done through the Basic Local Alignment



Search Tool (BLAST) in GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>) and the MalAvi database (<http://mbio-serv2.mbioekol.lu.se/Malavi/>; Bensch et al. 2009).

## Results

We obtained blood samples from a total of 194 Cattle Egrets and 127 Smooth-billed Anis from Santa Cruz and Isabela islands (Table 1). Of the 194 Cattle Egrets, 35 were nestlings that were very close to fledging; they were able to walk around the mangroves and stretch their wings fully but not fly away yet. From 2003-2009 we sampled 56 dead Smooth-billed Anis from which we obtained blood and liver tissue samples. From 2013-2015 we obtained other organ tissue (heart and/or liver, kidney and lung) samples from 2 egrets and 8 anis from Santa Cruz and 1 Smooth-billed Ani from Isabela. All samples tested negative for Haemosporidian parasites (*Plasmodium* spp. and *Haemoproteus* spp.) during our initial parasite screening resulting. Re-testing all 2013-2015 samples three times revealed two egrets and one ani positive for *Haemoproteus multipigmentatus*. *H. multipigmentatus* lineages ZEGAL05 and ZEGAL07 (Valkiūnas et al. 2010, Levin et al. 2011) were retrieved from the positive amplicons. Microscopic examination of Smooth-billed Ani (n=20) and Cattle Egret (n=20) smears revealed no evidence of any erythrocytic stages of development of Haemosporidian parasites. Overall, prevalence of *Plasmodium* spp. for egrets and anis was 0% (CI 0-0.024, 0-0.037 respectively), and prevalence of *Haemoproteus multipigmentatus* was 0.01% (CI 0.002-0.04) for egrets and 0.008% (CI 0.0004-0.05) for anis.

**Table 1.** Cattle Egret (*Bubulcus ibis*) and Smooth-billed Ani (*Crotophaga ani*)

individuals captured per island, year, month and time of day.

	2003-2009			2013			2014				2015			Total
	Jul	Nov	Dic	Jun	Jul	Aug	Feb	Mar	Apr	May	Apr	May	Jun	
<b>Egrets</b>				1		1	20	8	14	15	24	99	12	194
<b>Isabela</b>														
am									7		98			105
pm									7					7
<b>Sta. Cruz</b>														
am				1									12	13
pm						1	20	8		15	24	1		69
<b>Anis</b>	7	53	3	3	1		3	6			8	24	19	127
<b>Isabela</b>														
am					3			1	6		7	8		25
pm											13	6		19
<b>Sta. Cruz</b>														
am			53	3				2			5		1	64
pm						1					3		4	8
na	7											4		11

Egrets were found to be heavily ectoparasitized by louse flies (Hippoboscidae), particularly when captured from their roosting sites in the agricultural zone. We sampled a total of 167 louse flies, in pools of 1-7 individuals per each of 12 egrets in Santa Cruz and 57 egrets in Isabela island. All flies tested negative for Haemosporidian parasites (*Plasmodium* spp. and *Haemoproteus* spp.). Additionally, we captured a total of 79 female mosquitoes in CDC traps at the organic landfill in Isabela in 2013, 69 Black Salt Marsh Mosquitos and 10 Southern House Mosquitos. In the same year, a total of 91 female Black Salt Marsh Mosquitos were captured in light traps at the egret roosting site in Isabela. In the following year (2014) and at the same site, we captured 455 Southern House Mosquito females in light traps and zero Black Salt Marsh Mosquitos. In 2014, a total of 3, 096 female Black Salt Marsh Mosquitos were captured at the organic landfill in Isabela; 3, 079 females were

captured in light traps and 17 females were captured using gravid traps. Southern House Mosquito captures totaled 13 female mosquitoes at this same site in 2014 captured in gravid traps. Also in 2014, we captured a total of 17 female Black Salt Marsh Mosquitos and 5 female Southern House Mosquitos at Punta Estrada, Santa Cruz. In total, we subjected 3, 756 female mosquitoes captured in 2013 and 2014 in Santa Cruz and Isabela which consisted of 483 Southern House Mosquitos and 3, 273 Black Salt Marsh Mosquitos through molecular disease screening. We found all head/thorax and abdomen regions of female mosquitoes to be negative for Haemosporidian parasites (*Plasmodium* spp. and *Haemoproteus* spp.).

## **Discussion**

We found no evidence that the Cattle Egret or the Smooth-billed Ani play a role as reservoir hosts for *Plasmodium* spp. Although we found three introduced birds positive for *Haemoproteus multipigmentatus*, there was no indication of development of the parasite in the blood of these introduced birds. The prevalence of *H. multipigmentatus* in these bird populations is too low to consider them reservoir hosts; thus, this is likely another example of spillover from Galapagos Doves. Furthermore, we did not find evidence of *H. multipigmentatus* in the louse flies obtained from Cattle Egrets, despite how heavily ectoparasitized they were.

Most studies that have reported screening for haemosporidian parasites in Cattle Egrets and Smooth-billed Anis have had results similar to ours (Woodworth et al. 1989, Telford et al. 1992, Lima et al. 2010, Larcombe and Gauthier 2015). However, these studies had sample sizes of only one to 16 individuals, with the exception of Larcome and Gauthier (2015) which sampled 40 Cattle Egret nestlings in the Camargue, Southern France. This study tested for the presence of avian malaria in

160 nestlings of Little Egrets (*Egretta garzetta*), Cattle Egrets, Black-crowned Night Herons (*Nycticorax nycticorax*) and Squacco Heron (*Ardeola ralloides*) and found no evidence of malaria infection. They did find four lineages of *Plasmodium* spp. in Common House Mosquitoes (*Culex pipiens*) which they interpreted as exhibiting suitable characteristics for the transmission of disease and argue that perhaps these colonial herons have evolved some physiological or behavioral strategy to resist *Plasmodium* infection. On the other hand, Ferraguti et al. (2013) found two *Plasmodium* lineages and one *Haemoproteus* lineage in three of 13 Cattle Egrets sampled in southwestern Spain.

To our knowledge, the population size of Cattle Egrets has not been estimated in the Galapagos Islands. However, breeding occurs at few specific mangroves and daily migrations from roosting/breeding sites are made to feeding sites in the highlands (Phillips et al. 2012), and we identified and sampled egrets at roosting sites, breeding sites and foraging sites on both islands. The last estimates of ani population size were 4800 and 100 individuals in the farm zones of Santa Cruz and Isabela islands, respectively (Rosenberg et al. 1990). On Isabela, we sampled individuals from every ani territory surrounding the town of Puerto Villamil, providing a good representation of the ani population in this area, that was of special interest due to findings of positive passerines around this location in previous surveys (Levin et al. 2013). Thus, we are confident that our sample is representative of the populations of Smooth-billed Anis and Cattle Egrets on Santa Cruz and southern Isabela islands and our results suggest that the true population prevalence in these areas is close to zero.

Furthermore, prevalence of mosquito-borne parasites such as *Plasmodium* spp. has been shown to vary throughout the year, particularly in temperate zones

(Cosgrove et al. 2008, Atkinson and Samuel 2010). And although transmission in the tropics occurs year-round (Valkiūnas 2005), prevalence is influenced by the effects of temperature and precipitation on mosquito dynamics and availability of larval habitat (Samuel et al. 2011) and has been found to be higher in the wet season (Chasar et al. 2009). The Galapagos Islands exhibit two main seasons marked by changes in temperature and precipitation. The hot season, characterized by high temperatures and irregular rainfall, commonly occurs from January to May. The cool season occurs from June to December, with no rainfall in the lowlands, consistent mist in the highlands and lower temperatures all throughout (Trueman and d'Ozouville 2010). Although we did not sample all months of the year, we were able to obtain blood samples from introduced birds in both seasons (Table 1). However, the bulk of captures were obtained around the wet season in 2015, the sampled year with more precipitation, likely increasing our probabilities of sampling infected birds if there had been any. We also obtained blood samples from introduced birds captured at different times during the day, widening our window of opportunity to detect the parasite in its blood stage. No evidence of *Plasmodium* spp. parasites in vector mosquitoes in proximity to introduced birds further suggests that these are not the reservoir hosts. However, the dry sampling periods that are non-conducive for mosquito breeding could have lowered the chances of detecting the parasite.

Cattle Egrets have not been documented to have any large-scale impacts on Galapagos wildlife and this study was the first to successfully capture live Cattle Egrets in the archipelago. Their recognition as introduced species due to their association with cattle has made them a target of management efforts; however, the negative impacts of egrets on the endemic wildlife, if any, should be further investigated. Smooth-billed Anis, on the other hand, have been suggested to be an

ecosystem threat as seed dispersers of the highly invasive Mysore Raspberry, *Rubus niveus* (Connett et al. 2013). Connett et al.'s analysis of an gizzard content revealed that Mysore Raspberry was the second most abundant seed in its diet and a Darwin's finch nestling was also recovered. This illustrates two important negative effects on the endemic wildlife; however, further research about the biology of these birds is needed.

We have no evidence of the involvement of these two introduced birds as reservoirs of *Plasmodium* spp. in southern Isabela and Santa Cruz islands. Further research will be needed to find the reservoirs and describe the dynamics that maintain the parasite in the islands. It remains unclear how this parasite arrived to the archipelago and how it is being maintained but now we understand that future research should perhaps focus on other bird species or other islands. Migratory or resident shorebirds found in close proximity with Galapagos Penguins, the species which is most consistently found with *Plasmodium* spp. infections (Levin et al. 2009, 2013), would be good candidates to begin with.

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### **3. Avian blood parasites (Haemosporida) in Galapagos: prevalence estimates that account for imperfect detection of the PCR test**

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## Abstract

Avian haemosporidian parasites are nearly ubiquitous as they are found in bird populations all around the globe. Their ability to infect a broad range of hosts is cause for conservation concern in tropical archipelagos where they are unusually uncommon. Few haemosporidians are known to occur in the Galapagos Islands, of which only *Haemoproteus multipigmentatus* and a *Plasmodium* species have been found in multiple host species in the archipelago, including passerines. In this study, we use site-occupancy modeling to estimate blood parasite prevalence and the factors that influence parasite detection and prevalence of each of these two parasites. By accounting for imperfect detection of the diagnostic PCR test, we found that the prevalence estimates for *H. multipigmentatus* and *Plasmodium* sp. were considerably higher (0.21, CI 0.18-0.26 and 0.27, CI 0.15-0.45, respectively) than prevalence estimates when samples were tested just once (0.14 and 0.11, respectively). As we expected, due to known differences in parasitemia, the probability of detection was higher for *H. multipigmentatus* (0.66, CI 0.59-0.71) than for *Plasmodium* sp. (0.35, CI 0.17-0.59). Species group, based on taxonomic family and foraging habits, and number of PCR runs were important detection covariates for *H. multipigmentatus* and *Plasmodium* sp., respectively. Species group, sampling year, and location were important factors associated with the estimates of prevalence of *H. multipigmentatus* but no important covariates were identified for *Plasmodium* sp. prevalence. We obtained estimates of prevalence of avian haemosporidians for bird hosts in the Galapagos archipelago that take into account uncertainty in the detection of the diagnostic test and the estimation process. Previous prevalence estimates for both parasites underestimated the extent of exposure or infection and hence biased low our assessment of the risk of parasitism for the avifauna of Galapagos.

## Introduction

Avian haemosporidians (*Plasmodium*, *Haemoproteus* and *Leucozytozoon*) are a highly diverse group of parasites with a worldwide distribution (Valkiūnas 2005, Bensch et al. 2009). Their life cycles may differ among genera and species but they all multiply asexually in avian hosts and undergo sexual reproduction in Dipteran vectors. Avian haemosporidians reveal a higher diversity in continental tropics while their diversity appears to be limited in tropical archipelagos (Clark et al. 2014). The ability of these parasites, particularly those in the genera *Plasmodium* and *Haemoproteus*, to infect a broad range of hosts (e.g. Waldenström et al. 2002, Ricklefs et al. 2004, Fallon et al. 2005, Beadell et al. 2006, Ishtiaq et al. 2007) causes conservation concern in tropical archipelagos where the parasites are not common or are virtually absent because hosts are thought to be immunologically naïve.

*Plasmodium relictum* in Hawaii, for example, infected many species of native and introduced Hawaiian birds after its introduction to the archipelago and is implicated in the decline and extinction of many endemic honeycreepers (Drepanididae) (Warner et al. 1968, van Riper et al. 1986). Furthermore, host-switching of the parasites between closely related bird species appears to be common in the evolutionary history of avian haemosporidian parasites (Ricklefs and Fallon 2002). Spillover events have also been reported in the recent literature, finding these parasites infecting species that are not necessarily closely related to typical hosts (e.g. Moens et al. 2016, Chagas et al. 2017, Jaramillo et al. 2017).

In the Galapagos Islands, Ecuador, *Haemoproteus iwa* has been found in frigatebirds (*Fregata* spp.) (Levin et al. 2011), *Haemoproteus jenniae* has been detected in swallow-tailed gulls (*Creagrus furcatus*; Levin et al. 2012) and

*Haemoproteus multipigmentatus* in Galapagos doves (*Zenaida galapagoensis*; Santiago-Alarcon et al. 2008). *H. multipigmentatus* was presumably introduced with domestic rock pigeons (*Columba livia*) and remains in the endemic Galapagos dove even after the eradication of domestic pigeons (Santiago-Alarcon et al. 2008, Valkiunas et al. 2010). *H. multipigmentatus* spills over from doves to several native (Jaramillo et al. 2017) and two introduced birds in the archipelago, cattle egrets (*Bubulcus ibis*) and smooth-billed anis (*Crotophaga ani*; Chapter 2). The only *Plasmodium* sp. known to occur in the islands was first reported in the Galapagos penguin (*Spheniscus mendiculus*), and subsequently in yellow warblers (*Setophaga petechia*), small ground finches (*Geospiza fuliginosa*) and a medium ground finch (*Geospiza fortis*) (Levin et al. 2009, 2013). This parasite has yet to be formally described to the species level due to a lack of evidence of its erythrocytic stages of development (Levin et al. 2009, 2013).

A better understanding of the parasites' ecology in the archipelago could provide enhanced information about the threat these parasites pose to the avifauna of Galapagos. However, Levin et al.'s (2013) molecular screening showed that the proportion of positive samples testing positive again was very low, indicating imperfect pathogen detection by the diagnostic test. Estimates of prevalence for *Plasmodium* sp. and *H. multipigmentatus* made by testing individuals only once are therefore likely underestimates of the actual proportion of infected birds in the archipelago. Determining true infection status is challenging in studies of free-ranging wildlife because we generally lack a) a 'gold standard' test that reflects true infection status, b) multiple diagnostic methods to confirm infection, and/or c) prior knowledge of population prevalence (Lachish et al. 2012).



In haemosporidian studies pathogen detection is often imperfect and parasite prevalence varies with the diagnostic method. In contrast to molecular screening, prevalence is generally biased low with visual screening of blood smears, and even with molecular methods the relative prevalence of *Haemoproteus* and *Plasmodium* varies with different PCR approaches (Waldenström et al. 2004). Intensities of *Plasmodium* infection in blood are lower than those of *Haemoproteus*, which makes it more difficult to detect on blood smears (Fallon and Ricklefs 2008). Additionally, a molecular study of *Plasmodium* spp. in blood samples from wild blue tits (*Cyanistes caeruleus*) demonstrated that qPCR detection probabilities were strongly dependent on host parasite load (infection intensity; Lachish et al. 2012). Thus, the detection probability of a diagnostic test is likely specific to the parasite species and associated to levels of blood parasitemia in the host.

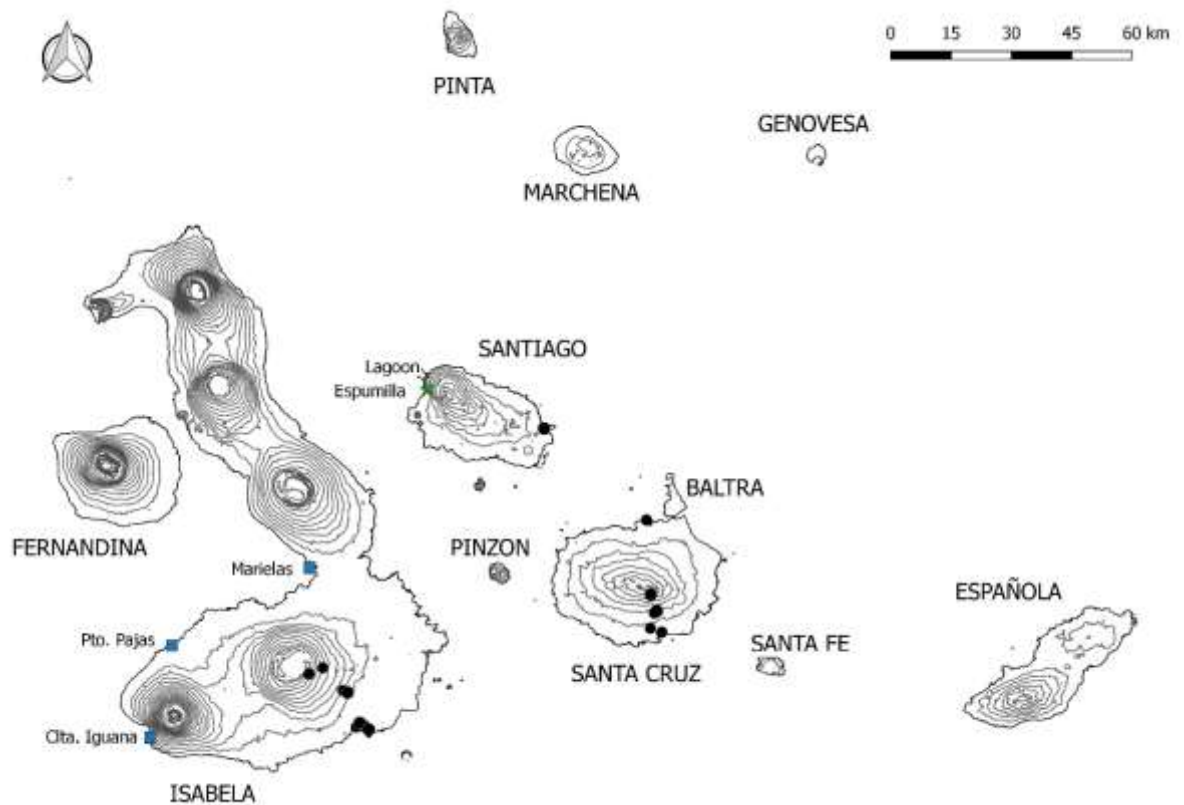
Occupancy approaches are useful in wildlife disease ecology because they acknowledge imperfect detection and account for this uncertainty in parameter estimates of prevalence (McClintock et al. 2010). These approaches use a history of multiple surveys or visits to a site (e.g. a patch of habitat), in which the presence or absence of a species of interest is recorded, and these ‘encounter histories’ are used to estimate the probabilities of detection and occupancy of the species (MacKenzie et al. 2002). In our study, the species of interest are the blood parasites: *Haemoproteus multipigmentatus* and *Plasmodium* sp., and a blood sample from a Galapagos bird is a ‘site’ that we ‘visit’ or test three times to create a parasite encounter history. Occupancy then corresponds to the prevalence of the parasite in avian hosts in the archipelago of Galapagos and detection probability is a measure of the sensitivity of the PCR diagnostic test.

Thus, we sought to obtain estimates of the prevalence of infection of *Plasmodium* sp. and *Haemoproteus multipigmentatus* in Galapagos native birds using an occupancy estimation approach. We expected estimates of prevalence of *Plasmodium* sp. and *H. multipigmentatus* would be higher than naïve estimates of prevalence when samples are tested just once, due to imperfect detection of parasite DNA by the PCR test. Additionally, due to general differences in parasitemia, we expected better detection for *H. multipigmentatus* than *Plasmodium* sp. Lastly, we used site-occupancy models to investigate factors that could be associated with estimation of the detection and occupancy (prevalence) parameters of each parasite.

## **Methods**

We sampled land birds along an altitudinal gradient on Santa Cruz, Isabela, and Santiago Islands during 2013-2015 (Figure 1). We used 2.5-3m tall, 6-9m wide, mist nets for capture of land birds and banded most birds with an aluminum band with a unique identification number. Morphological measurements were taken and a blood sample of volume equivalent to less than 1% of each bird's body weight (Owen 2011) was collected via brachial venipuncture. Samples were centrifuged and red blood cells were placed in Longmire's lysis buffer for molecular analysis (Longmire et al. 1988). Galapagos penguin blood samples were obtained by a joint team of researchers from the Saint Louis Zoo and the Charles Darwin Research Station in November 2016 on southwestern Isabela.

**Figure 1. Map of sampling locations in the Galapagos Islands, Ecuador.** Blue squares indicate three sites included in *Plasmodium* sp. analyses. Green stars indicate the two sites included in *Haemoproteus multipigmentatus* analyses. Black circles are all other land bird sampling locations.



We extracted DNA from the land bird samples using a standard phenol-chloroform protocol (Sambrook et al. 1989), with a final dialysis step in 1M Tris pH8, 5M NaCl, 0.5M EDTA, dH2O solution, at the University of Missouri – Saint Louis (UMSL). We extracted Galapagos penguin samples using the animal blood protocol of DNeasy® Blood and Tissue Kits (QIAGEN Group) at the Galapagos Biosecurity Agency’s (ABG) molecular laboratory in Galapagos. Parasite screening involved amplifying a region of the parasite’s mitochondrial cytochrome *b* gene following the nested PCR reaction in Waldenström et al. (2004). Positive and negative controls were always used; the positive controls were a sequenced *Plasmodium*-positive Galapagos penguin and/or a sequenced *Haemoproteus*-positive Galapagos dove that

amplified consistently, and the negative control consisted of all PCR reagents without template DNA. Amplified parasite DNA was sequenced either following Levin et al. (2011) in an ABI 3130 automated sequencer at the University of Missouri – Saint Louis or by Eurofins Genomics (12701 Plantside Drive Louisville, Kentucky 40299). Identification of sequences was done by comparison with existing sequences (Blast) in the MalAvi database (<http://mbio-serv2.mbioekol.lu.se/Malavi/>; Bensch et al. 2009).

Each bird DNA sample was tested three times for the detection of *H. multipigmentatus* and *Plasmodium* sp. If parasite DNA was detected (amplified) for any given sample, the amplicon was sequenced for parasite identification. For each testing event (replicate), a bird was marked as positive (1) or negative (0) depending on whether the parasite was detected. From these, a detection history was obtained for each bird. Naïve prevalence was calculated as the number of birds that tested positive in the first PCR replicate divided by the total number of birds tested. Occupancy analyses were done in RStudio Version 1.1.383 (RStudio Team 2016) using the R package ‘unmarked’ (Fiske and Chandler 2011). The analyses to estimate prevalence of *Plasmodium* sp. included 58 penguins sampled in three locations around southwestern Isabela in 2016. The analyses to estimate prevalence of *H. multipigmentatus* included 484 individuals of 10 passerine species from two locations on the island of Santiago that were sampled in three consecutive years (2013-2015). Only three individuals were recaptured once; one capture event from each recapture was randomly selected and removed from further analysis. Doves were excluded from the analysis because of their small sample size and prevalence and detection equal to 1, making it difficult to parse sources of variation.

Depending on the parasite, the influence of various covariates was evaluated on the estimation of occupancy and detection of parasites in birds, based on the following hypotheses:

For *H. multipigmentatus*, 1) Prevalence will vary by year of sampling because weather conditions related to louse fly vector abundance varied. Alternatively, prevalence in passerines might be influenced by the abundance of the reservoir host (Galapagos doves; Jaramillo et al. 2017) and thus will vary by year because the abundance of doves varied. Along the same line of thought, 2) prevalence in passerines will vary by sampling location because abundance of doves varied by location (Jaramillo et al. 2017). 3) Prevalence would vary by group of species, based on their taxonomic family and foraging habits, where ground finches and mockingbirds will have higher prevalence because they forage on the ground and in closer proximity to doves. Alternatively, mockingbirds will have higher prevalence because vectors may come into contact with birds that are more similar in size to the dove reservoir host. 4) Prevalence will vary by sex because sex-specific hormones may influence the host immune system, with males being more likely to be infected by avian haemosporidians than females (Calero-Riestra and García 2016). Lastly, 5) detection will vary between groups of species because the parasite may be able to develop/multiply in some groups more than others, thus, how much parasite DNA is present to detect would vary by group.

For *Plasmodium* sp., 1) prevalence will vary with sampling location because variation in climatic conditions across locations may influence the abundance of the mosquito vector. 2) Prevalence will vary by age, with juvenile penguins being more susceptible to infection than adults because they are immunologically naïve (Graczyk

et al. 1994). Lastly, 3) detection will vary by run because technique will improve with number of PCR runs performed.

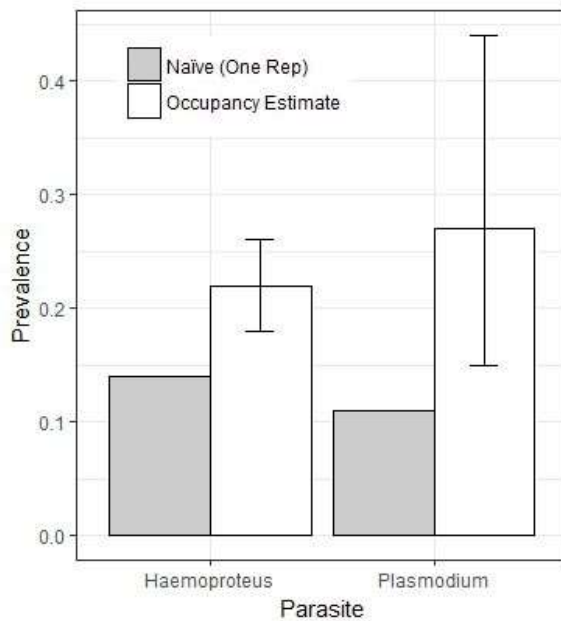
We used corrected Akaike Information Criteria (AICc) and  $\Delta$ AICc values to estimate relative support for the models (Burnham and Anderson 2002). Due to a lack of sexual dimorphism in some species a separate analysis was done with a subset of the data with individuals of known sexes. We tested all possible combinations of covariates in three models set: *H. multipigmentatus* in passerines (Table 1), *H. multipigmentatus* in passerines, including a sex effect (Table 3), and *Plasmodium* sp. in penguins (Table 4). The relative importance of each occupancy covariate was calculated using the sum of the AICc weights across all models containing the predictor variables and parameter estimates were derived from model averaging.

## Results

We sampled 2031 individuals from 17 species of native land birds on Santa Cruz, Isabela, and Santiago islands. Land birds were found infected with *Haemoproteus multipigmentatus* almost exclusively on the island of Santiago. Out of 484 individuals tested for this analysis, 67 were PCR-positive for *H. multipigmentatus* in the first PCR replicate, 0.14 naïve prevalence. The estimate of prevalence for *H. multipigmentatus* was 0.21 (CI 0.18-0.26; Figure 2), from the highest-ranking model in which occupancy was constant (Table 1). The detection probability of the diagnostic PCR test was 0.66 (CI 0.59-0.71), from the highest-ranking model in which detection was constant (Table 1). Detection varied with group, averaging  $0.58 \pm 0.13$  for flycatchers,  $0.64 \pm 0.04$  for ground finches,  $0.73 \pm 0.08$  for mockingbirds,  $0.50 \pm 0.17$  for tree finches and  $0.71 \pm 0.1$  for warblers, from model averaging the *H. multipigmentatus* occupancy model set (Table 1).

**Figure 2. Prevalence estimates for Haemosporidian parasites in Galapagos.**

Prevalence estimates are shown for naïve (grey bars) and occupancy estimates (clear bars) for *Haemoproteus multipigmentatus* and *Plasmodium* sp. The error bars for the occupancy estimates represent 95% confidence intervals.



**Table 1.** Candidate models for estimating occupancy ( $\Psi$ ) and the probability of detection ( $p$ ) of *Haemoproteus multipigmentatus* in Santiago passerines. We explored potential variation in occupancy due to sampling year (year), location, and species group (group); and variation in detection due to species group. The number of parameters (K), small sample size-corrected Akaike information criterion values (AICc and  $\Delta$ AICc), Akaike weights ( $w$ ) and log-likelihoods (LL) are shown for each model.

Model	K	AICc	$\Delta$ AICc	$w$	LL
$\Psi_{year+location+group} P_{group}$	13	834.62	0.00	0.38	-403.92
$\Psi_{year+location+group} P.$	9	834.86	0.24	0.34	-408.24
$\Psi_{year+group} P_{group}$	12	837.50	2.88	0.09	-406.42
$\Psi_{year+group} P.$	8	837.74	3.11	0.08	-410.72
$\Psi_{location+group} P.$	7	839.55	4.92	0.03	-412.66
$\Psi_{location+group} P_{group}$	11	839.67	5.04	0.03	-408.55
$\Psi_{group} P.$	6	841.71	7.08	0.01	-414.77
$\Psi_{group} P_{group}$	10	841.76	7.14	0.01	-410.65
$\Psi_{year+location} P.$	5	842.50	7.87	0.01	-416.19
$\Psi_{year+location} P_{group}$	9	842.51	7.88	0.01	-412.06
$\Psi_{year} P.$	4	844.07	9.45	0.00	-417.99
$\Psi_{year} P_{group}$	8	844.20	9.58	0.00	-413.95
$\Psi_{location} P_{group}$	7	850.92	16.29	0.00	-418.34
$\Psi_{location} P.$	3	851.25	16.62	0.00	-422.60
$\Psi. P_{group}$	6	851.28	16.66	0.00	-419.55
$\Psi. P.$	2	851.54	16.92	0.00	-423.76

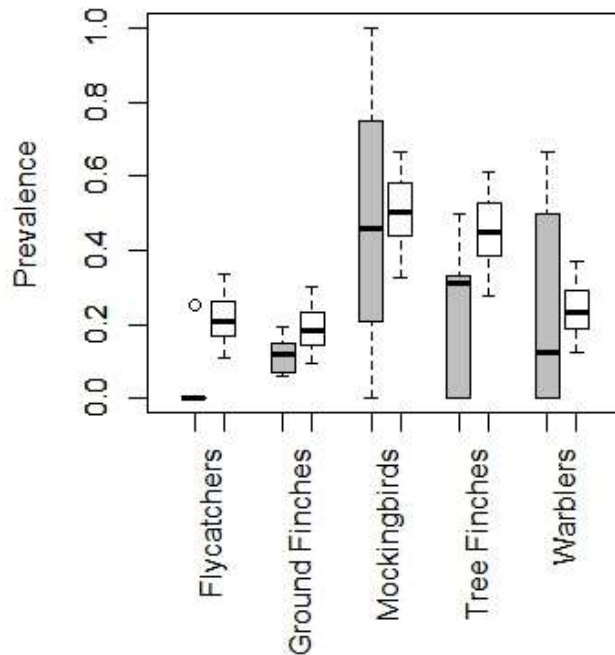
The best model for estimating occupancy and detection of *H. multipigmentatus* has a weight of 0.38 suggesting substantial model selection uncertainty (Table 1). The measures of relative importance of the occupancy predictor variables were 0.98, 0.92 and 0.80 for species group, year, and location, respectively. Species-group specific prevalence estimates are provided in Table 2 and Figure 3, year and location prevalence estimates are provided in Figure 4, by model-averaging over the *H. multipigmentatus* occupancy model set that excludes the sex covariate (Table 1). The relative importance of the predictor variables from the *H. multipigmentatus* model set that included a sex effect (Table 3) were 0.77, 0.71, 0.69, and 0.27 for year, location, species group, and sex, respectively. Naïve prevalence was 0.17 for males and 0.11 for females while prevalence estimates, averaged from the occupancy model set with known sexes (Table 3), were  $0.27 \pm 0.11$  for males and  $0.28 \pm 0.11$  for females.

**Table 2.** Naïve and estimated prevalence for *Haemoproteus multipigmentatus* in passerine species groups on Santiago island, Galapagos (mean  $\pm$  SE). Naïve prevalence was calculated as the number of birds positive for *H. multipigmentatus* in the first PCR replicate divided by the total number of birds tested. Estimated prevalence was model-averaged across the entire occupancy model set.

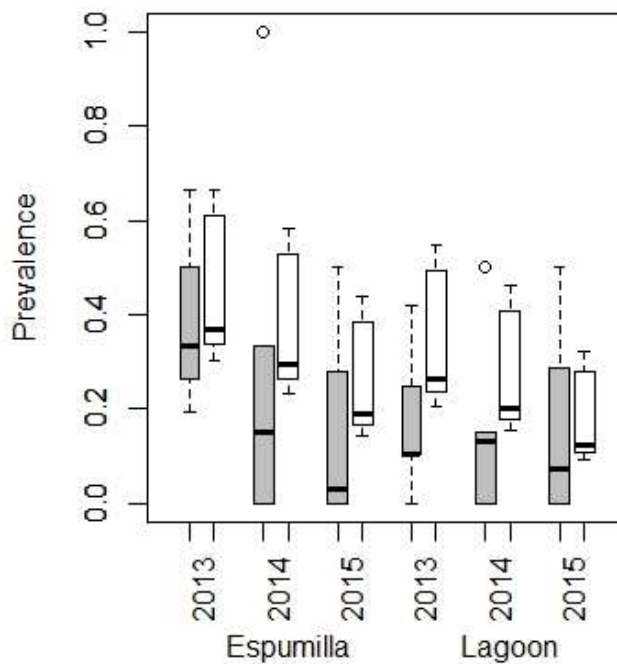
Species Group	n	Naïve prevalence	Estimated prevalence $\Psi$
<b>Tree finches (Thraupidae)</b>	20	0.25	0.45 (0.18)
<i>Camarhynchus parvulus</i>			
<i>C. psittacula</i>			
<i>Platyspiza crassirostris</i>			
<b>Ground finches (Thraupidae)</b>	352	0.11	0.19 (0.05)
<i>Geospiza fuliginosa</i>			
<i>G. fortis</i>			
<i>G. magnirostris</i>			
<i>G. scandens</i>			
<b>Warblers (Parulidae)</b>	59	0.15	0.24 (0.08)
<i>Setophaga petechia</i>			
<b>Flycatchers (Tyrannidae)</b>	28	0.07	0.22 (0.09)
<i>Myiarchus magnirostris</i>			
<b>Mockingbirds (Mimidae)</b>	25	0.44	0.50 (0.13)
<i>Mimus parvulus</i>			



**Figure 3.** Naïve (grey boxplots) and estimated prevalence (clear boxplots) for *Haemoproteus multipigmentatus* in various infected species groups on Santiago island. Naïve prevalence was calculated as the number of birds positive for *H. multipigmentatus* in the first PCR replicate by the total number of birds tested. Estimated prevalence was model-averaged across the entire occupancy model set.



**Figure 4.** Naïve (grey boxplots) and estimated prevalence (clear boxplots) for *Haemoproteus multipigmentatus* in the three sampling years (2013-2015) and two locations (Espumilla and Lagoon) on Santiago island. Naïve prevalence was calculated as the number of birds positive for *H. multipigmentatus* in the first PCR replicate divided by the total number of birds tested. Estimated prevalence was model-averaged across the entire occupancy model set.



**Table 3.** Candidate models for estimating occupancy ( $\Psi$ ) and detection of *Haemoproteus multipigmentatus* in Santiago passerines with known sexes. We explored potential variation in occupancy due to sampling year (year), location, species group (group), and sex; and variation in detection due to species group. The number of parameters (K), small sample size-corrected Akaike information criterion values (AICc and  $\Delta$ AICc), Akaike weights ( $w$ ) and log-likelihoods (LL) are shown for each model.

Model	K	AICc	$\Delta$ AICc	$w$	LL
$\Psi_{year+location+group}$ Pgroup	9	671.22	0.00	0.19	-326.38
$\Psi_{year+location+group}$ P.	7	672.89	1.67	0.08	-329.31
$\Psi_{year+group}$ Pgroup	8	672.99	1.77	0.08	-328.31
$\Psi_{year+location}$ Pgroup	7	673.18	1.96	0.07	-329.45
$\Psi_{year+location+group+sex}$ Pgroup	10	673.21	1.99	0.07	-326.33
$\Psi_{year+location}$ P.	5	673.73	2.51	0.06	-331.79
$\Psi_{location+group}$ Pgroup	7	674.01	2.79	0.05	-329.86
$\Psi_{year+group}$ P.	6	674.64	3.42	0.04	-331.21
$\Psi_{year+location+group+sex}$ P.	8	674.89	3.67	0.03	-329.26
$\Psi_{year+group+sex}$ Pgroup	9	675.07	3.85	0.03	-328.31
$\Psi_{year+location+sex}$ Pgroup	8	675.19	3.97	0.03	-329.41
$\Psi_{group}$ Pgroup	6	675.26	4.04	0.03	-331.53
$\Psi_{location+group}$ P.	5	675.37	4.15	0.02	-332.61
$\Psi_{year}$ Pgroup	6	675.50	4.28	0.02	-331.64
$\Psi_{location}$ Pgroup	5	675.54	4.32	0.02	-332.70
$\Psi_{year+location+sex}$ P.	6	675.74	4.52	0.02	-331.77
$\Psi_{location+group+sex}$ Pgroup	8	675.81	4.59	0.02	-329.72
$\Psi_{year}$ P.	4	675.95	4.73	0.02	-333.93
$\Psi_{location}$ P.	3	676.30	5.08	0.02	-335.12
$\Psi_{year+group+sex}$ P.	7	676.68	5.46	0.01	-331.20
$\Psi_{group}$ P.	4	676.69	5.47	0.01	-334.29
$\Psi.$ Pgroup	4	676.96	5.74	0.01	-334.43
$\Psi_{location+group+sex}$ P.	6	677.16	5.94	0.01	-332.47
$\Psi_{group+sex}$ Pgroup	7	677.33	6.11	0.01	-331.52
$\Psi_{location+sex}$ Pgroup	6	677.52	6.30	0.01	-332.66
$\Psi_{year+sex}$ Pgroup	7	677.54	6.32	0.01	-331.63
$\Psi.$ P.	2	677.65	6.43	0.01	-336.81
$\Psi_{year+sex}$ P.	5	677.96	6.74	0.01	-333.90
$\Psi_{location+sex}$ P.	4	678.28	7.06	0.01	-335.09
$\Psi_{group+sex}$ P.	5	678.74	7.52	0.00	-334.29
$\Psi_{sex}$ Pgroup	5	678.99	7.77	0.00	-334.42
$\Psi_{sex}$ P.	3	679.65	8.43	0.00	-336.80

Not a single *Plasmodium* sp. infection was found in any of the sampled Galapagos land birds during this survey in either Santa Cruz, Santiago or Isabela islands. Out of 57 penguins, 6 were PCR-positive for *Plasmodium* sp. in the first PCR replicate, 0.11 naïve prevalence. The estimated prevalence for *Plasmodium* sp. was 0.27 (CI 0.15-0.45) (Figure 2), from the highest-ranking model (Table 4). The highest-ranking occupancy model for *Plasmodium* sp. has a weight of 0.63, and the occupancy predictor variables had little importance, 0.24 for age and 0.12 for location (Table 6). The detection probability of the diagnostic PCR test was 0.35 (CI 0.17-0.59), from the highest-ranking model where detection was constant. Detection increased with increasing number of PCR runs, ranging from  $0.09 \pm 0.08$  to  $0.80 \pm 0.16$ , from model averaging the *Plasmodium* sp. occupancy model set (Table 4).

**Table 4.** Candidate models for estimating occupancy ( $\Psi$ ) and detection (P) of *Plasmodium* in Galapagos penguins. We explored potential variation in occupancy due to sampling location (location) and penguin's age (age), and variation in detection with number of PCR runs performed (run). The number of parameters (K), small sample size-corrected Akaike information criterion values (AICc and  $\Delta$ AICc), Akaike weights ( $w$ ) and log-likelihoods (LL) are shown for each model.

Model	K	AICc	$\Delta$ AICc	$w$	LL
$\Psi$ . <i>Prun</i>	3	96.22	0.00	0.63	-44.88
$\Psi_{age}$ <i>Prun</i>	4	98.50	2.28	0.20	-44.86
$\Psi_{location}$ <i>Prun</i>	5	100.39	4.16	0.08	-44.60
$\Psi$ . P.	2	101.32	5.10	0.05	-48.55
$\Psi_{location+age}$ <i>Prun</i>	6	102.88	6.66	0.02	-44.60
$\Psi_{age}$ P.	3	103.54	7.32	0.02	-48.55
$\Psi_{location}$ P.	4	105.34	9.12	0.01	-48.29
$\Psi_{location+age}$ P.	5	107.74	11.52	0.00	-48.28

## Discussion

In this study, we obtained estimates of prevalence of haemosporidian parasites, *Haemoproteus multipigmentatus* and *Plasmodium* sp. in Galapagos land birds and penguins, that account for imperfect detection of the PCR-based diagnostic assay. We

found that the prevalence estimates for *H. multipigmentatus* and *Plasmodium* sp. were considerably higher when accounting for imperfect detection (0.21, CI 0.18-0.26 and 0.27, CI 0.15-0.45, respectively) than naïve prevalence estimates when samples were tested just once (0.14 and 0.11, respectively) (Figure 2). If we had tested individuals only once we would have underestimated the number of infected individuals in the populations of land birds on Santiago and penguins on Isabela. By testing the samples three times and accounting for imperfect detection of the diagnostic assay, we have obtained more reliable estimates of prevalence for both parasites.

As we expected, the probability of detection was higher for *H. multipigmentatus* (0.66, CI 0.59-0.71) than for *Plasmodium* sp. (0.35, CI 0.17-0.59). We suspect this difference in test sensitivity is related to known differences in parasitemia exhibited by these parasites (Waldenstron et al. 2004, Fallon and Ricklefs 2008). However, information on infection intensities are not available in our study, given our PCR methods and also due to the lack of erythrocytic stages of development found in penguin and land bird's blood smears (Levin et al. 2009, 2013 and Jaramillo et al. 2017). Nonetheless, we are detecting parasite DNA in blood and the chances of finding each parasite's DNA are likely related to differences in host parasitic loads. Accounting for variation in detection with the number of PCR runs performed was important for the parameter estimation of *Plasmodium* sp. Unfortunately, variation in detection by run could not be considered with the *H. multipigmentatus* data set because PCR testing was done by order of year, whereas for *Plasmodium* sp. testing was random. However, it was important to consider variation in *H. multipigmentatus* detection between the different avian species groups.

Avian host species group was an important covariate for *Haemoproteus multipigmentatus* prevalence estimates (Sum of AIC weights 0.98). The passerine groups (flycatchers, ground finches, tree finches and warblers) exhibit variation in prevalence with mockingbirds having the highest prevalence of *H. multipigmentatus* followed by tree finches (Table 4, Figure 3). The high prevalence in mockingbirds could be explained by their proximity to doves, as they forage on the ground, or by having similar body size as doves and therefore being more likely to come in contact with dove louse flies. However, tree finches forage mostly above ground and most of the tree finches (18/20) were of the smallest species, *Camarhynchus parvulus*. Thus, perhaps the differences in prevalence between groups, in presence of doves, have more to do with how these birds' immune systems respond to parasitic infection.

Year of sampling (Sum of AIC weights of 0.92) and location (Sum of AIC weights of 0.80) were also important covariates for *Haemoproteus multipigmentatus* prevalence estimates. Variation in prevalence among years and locations may be explained by changes in movement patterns of Galapagos doves, which seem to move frequently around the archipelago (Santiago-Alarcon et al. 2006). Prevalence was slightly lower in Lagoon than in Espumilla and decreased from 2013 to 2015 in both locations (Figure 4). Consistent with this explanation, we captured 0.07 doves per hour in Espumilla and 0.03 doves per hour in Lagoon (Jaramillo et al. 2017). Moreover, in these two locations, doves captured decreased by year with 0.17 doves per hour in 2013, 0.1 doves per hour in 2014, and 0.05 doves per hour in 2015. Alternatively, variation by year may be explained by environmental factors that influence the biology of the Hippoboscid vector. Louse fly prevalence in bird hosts has been found to correlate positively with temperature and precipitation (Senar et al. 1994, Eeva et al. 2013). However, our sampling year 2015 was wetter (sum

precipitation 571.4 mm/year) than 2014 (249.7 mm/year) and 2013 (246.5 mm/year) and 2015 was a slightly warmer year (avg. mean air temperature  $25.98^{\circ}\text{C} \pm 0.07$ ) than 2014 ( $25.19^{\circ}\text{C} \pm 0.1$ ) and 2013 ( $23.57^{\circ}\text{C} \pm 0.12$ ). Thus, the decrease in prevalence in 2015 may be more related to the population dynamics of Galapagos doves, the reservoir host, than to environmental factors affecting the louse fly vector.

Due to the lack of sexual dimorphism in several of some avian host species, we did not always differentiate males from females. Thus, we tested for sex-specific variation in a subset of the land bird data that included only ground finches, tree finches and warblers (n=405). However, sex was the least important variable in explaining variation in *H. multipigmentatus* prevalence estimates (Sum of AIC weights of 0.30). Although naïve prevalence is lower for females (0.1) than for males (0.14), estimates of prevalence from averaging the occupancy model set show no differences between the sexes ( $0.28 \pm 0.1$  for females and  $0.27 \pm 0.1$  for males). Thus, acknowledging imperfect detection also illustrated the lack of sex-specific differences in *H. multipigmentatus* prevalence in these three species groups.

Naïve indices of *H. multipigmentatus* prevalence for all species groups, years, and locations were often biased low in relation to the estimates of prevalence from the occupancy models, underestimating the extent of exposure or infection. Additionally, and perhaps more importantly, the estimates of prevalence were generally more precise when accounting for imperfect detection (Figure 3 and 4). This gain in precision in our estimates of prevalence may have implications not only for further studies of avian blood parasites in the Galapagos islands but for the conservation of their unique avifauna.

There was little support for differences in *Plasmodium* sp. prevalence estimates between adult and juvenile penguins or between the three locations sampled on Isabela Island. However, we must consider that there were about five times more adults than juveniles in our relatively small sample. Differences in prevalence between ages have been reported for the African black-footed penguins (*Spheniscus demersus*), juveniles being more prone to infection and mortality (Graczyk et al. 1994). Thus, it would be important to continue monitoring prevalence of *Plasmodium* sp. in juvenile and adult Galapagos penguins while acknowledging uncertainty in the detection method. Additionally, we suspect that the frequent movement of Galapagos penguins throughout their distribution in the archipelago, inferred by their high levels of gene flow (Nims et al. 2008), may keep prevalence of *Plasmodium* sp. fairly homogeneous among sampling locations.

Contrary to our expectations, based on Levin et al.'s (2013) findings of passerines positive for *Plasmodium* sp. via PCR on southern Isabela and Santa Cruz islands, we found no evidence of *Plasmodium* sp. in the land birds of Santa Cruz, Isabela, or Santiago islands in years 2013 to 2015. These results suggest that the prevalence of *Plasmodium* sp. in land birds is essentially zero and that Levin et al.'s (2013) findings reflected an outbreak at that time. However, it is possible that the rather dry sampling years 2013 and 2014 may have hindered *Plasmodium* sp. transmission by vectors. And even though our sampling year 2015 was wetter than the previous two years, a recent mosquito survey on the island of Santa Cruz found very low mosquito numbers during that year (Asigau et al. 2018). Thus, environmental factors may have not been favorable for *Plasmodium* sp. transmission in the past few years.

Using a site occupancy framework, we have obtained more reliable and precise estimates of prevalence of avian haemosporidians for bird hosts in the Galapagos archipelago. Previous prevalence estimates for both parasite taxa underestimated the proportion of birds exposed to or infected with the parasites and hence biased low our risk assessment of disease for the avifauna of Galapagos. Site-occupancy approaches could be applied in future research to estimate disease parameters that take into account biases in the sampling technique (e.g., mist netting), thus, further improving our assessment of blood parasite prevalence in the archipelago. Reliable assessments of disease prevalence are important not only for understanding epidemiology and the dynamics of wildlife disease (Jennelle et al. 2007, McClintock et al. 2010, Lachish et a. 2012), but can be critical for disease management and risk assessment of wildlife diseases.



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**4. Exploring the relationship between strength of the avian immune response and the time since arrival to the Galapagos archipelago**

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## **Abstract**

Life on islands is thought to be at great risk of introduced disease. Parasite communities in isolated islands are often small, reducing selective pressures that may result in weakening of the immune function of island hosts with evolutionary time. We investigated if the time since the arrival of a bird species to Galapagos correlates with the strength of its immune response. We found no indication that species that arrived to the islands more recently have stronger immune system function than earlier arrivals.

## **Introduction**

Isolated island parasite communities are often thought to be small and species poor (Wikelski et al. 2004), resulting in small number of host-parasite interactions and reduced selective pressures for island hosts (Hochberd and Moller 2001). Costs associated with maintaining the immune system (Sheldon & Verhulst 1996; Norris & Evans 2000) would favor reduced investment in immune function with time in parasite-poor communities (Jarvi et al. 2001, Van Riper & Scott 2001). Furthermore, the loss of genetic diversity in founder populations can result in low genetic variability at immunological loci that are required for effective resistance to disease (Bollmer et al. 2007, 2011; Whiteman et al. 2006, Sutton et al. 2011).

Most of the empirical evidence for the effect of introduced diseases on isolated wild avian populations comes from the introduction of avian pox virus and avian malaria (*Plasmodium relictum*) to Hawaii, which resulted in major extinctions of its avifauna (Warner 1968, Van Riper et al. 1986). Endemic honeycreepers experimentally infected with *P. relictum* have been shown to be highly susceptible to the pathogen (Jarvi et al. 2001). There are a few other examples of introduced disease

affecting isolated island populations (Tompkins and Gleeson 2006, Bunbury et al. 2008, Kerr 2012), but at the same time other evidence provides little support for the vulnerability of insular birds (Jarvi et al. 2003, Atkinson et al. 2006, Illera et al. 2008) or for simple hypotheses explaining their immune susceptibility (Matson 2006).

A thousand kilometers off the coast of Ecuador, the unique avifauna of Galapagos is still present today (Wikelski et al. 2004). However, some species are confined to extremely small populations in few locations (Fessl et al. 2010, Grant et al. 2000, Grant et al. 2005). Introduced pathogens and parasites represent some important risks and causes of mortality of birds in the archipelago (Vargas 1987, Fessl et al. 2006, Fessl and Tebbich 2002, Huber 2008, Kleindorfer et al. 2006, Santiago-Alarcon et al. 2008, O'Connor et al. 2010, Parker et al. 2011, Levin et al. 2013), establishing an urgent need to understand just how vulnerable these avian hosts are.

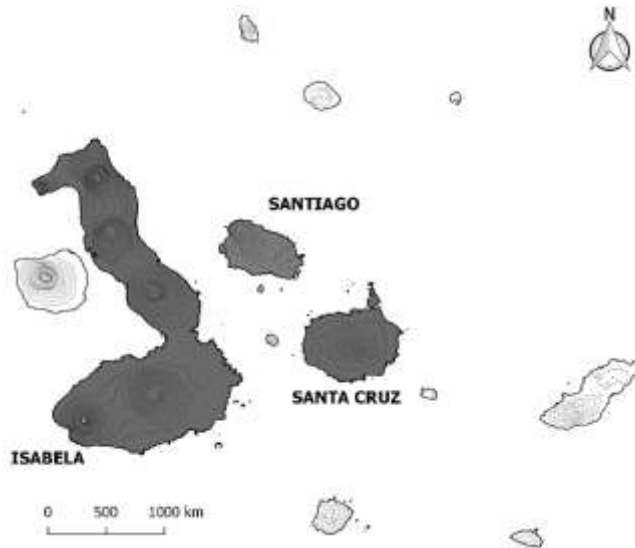
If the evolution of immune defenses is influenced by life in isolated islands with impoverished parasite communities, time since isolation from disease should increase the susceptibility of host species to pathogens. Here, we investigate the relationship between the strength of the avian immune response and the time since arrival to the Galapagos archipelago. We test the hypothesis that species that have arrived to the islands more recently, including introduced species, are more resistant to disease and present higher indices of the immune response than endemic species that arrived long ago.

## **Methods**

We captured birds on Santa Cruz, Santiago, and Isabela islands (Figure 1). Bird capture and blood sampling methods have been described in previous chapters. Blood samples were kept in heparinized capillaries at  $\sim 4^{\circ}\text{C}$  until centrifuged, within 6

hours of collection, after that serum was frozen at  $-4^{\circ}\text{C}$  in the field and at  $-80^{\circ}\text{C}$  in the laboratory at the University of Missouri – Saint Louis.

**Figure 1.** Map of the main islands of the Galapagos archipelago. Names are provided for islands included in the study (shaded).



Immune function comparisons were made for both native and introduced birds in the Galapagos Islands. Cattle egrets (*Bubulcus ibis*) and smooth-billed anis (*Crotophaga ani*) are introduced birds thought to have arrived sometime during the 1960's (Rosenberg et al. 1990, Wiedenfeld 2006). Molecular studies have estimated that yellow warblers (*Setophaga petechia*), Galapagos flycatchers (*Myiarchus magnirostris*), and Galapagos mockingbirds (*Mimus parvulus*) arrived or diverged approximately 0.27, 0.85 and 1.54 million years ago (mya), respectively (Chaves et al. 2012, Sari and Parker 2012, Stefka et al. 2011). Estimated time of arrival for Galapagos doves (*Zenaida galapagoensis*) is approximately 2mya and Darwin's finches (*Geospiza* spp.) are the earliest arrivals at 2.3mya (Johnson and Clayton 2000, Sato et al. 2001).

To characterize immune function, we used various indices that are applicable for studies of free-living wildlife and provide information about a range of protective



functions (Matson et al. 2005, Matson 2006). We quantified haptoglobin concentration ( $\text{mgml}^{-1}$ ) following the manual instructions of a commercially available assay (TP801; Tri-Delta Diagnostics, NJ, USA). For lysis and agglutination procedures, we followed Matson et al. (2005). Concentrations of several types of leucocytes were estimated from overall leukocyte counts (Werner 2001) and differential counts done by a technician in a veterinary diagnostic laboratory, AVL Veterinary Clinical Laboratory, St. Louis, MO.

To analyze the data, we used a conservative approach: phylogenetic generalized least squares (PGLS) (Symonds and Blomberg 2014), to account for inter-specific auto-correlation due to phylogenetic relationships (Felsenstein et al. 1985); and a liberal approach: generalized linear mixed effect models (GLMM) to allow for different error distributions and random effects. For all models, time of arrival was a continuous explanatory variable based on approximate time of arrival or date of diversification of the avian genus. The response variables tested were haptoglobin concentration, hemagglutination, hemolysis and heterophil, lymphocyte, and monocyte concentrations, tested independently. Total leukocyte concentration was excluded from the analysis due to correlation with the concentration of the different leukocyte types (Pearson's  $r \geq 0.46$ ). Correlation coefficients between all other variables were acceptable for analyses (Pearson's  $r \leq 0.34$ ). Eosinophil and basophil concentrations were not included in the analysis because they were nearly absent or completely absent. Only adult individuals were included in the analyses.

Statistical analyses were done in RStudio Version 1.1.383 (RStudio Team 2016). For PGLS analyses we used R packages 'ape' (Paradis and Strimmer 2004) and 'nlme' (Pinheiro et al. 2017). A majority rule consensus tree was built in

Mesquite Version 3.2 (Madison and Madison 2017) from a sample of 2000 trees based on a Hackett backbone from [www.birdtree.org](http://www.birdtree.org). For generalized linear mixed models we used R packages ‘lme4’ (Bates et al. 2015) and ‘glmmADMB’ (Fournier et al. 2012, Skaug et al. 2016) depending on the error distribution of the response variable. Time variables (year/month), spatial variables (island/location) and variables associated with an individual’s genus-level taxon were included as random factors in all GLMMs. Genus-level taxon was included as a random effect to account for the genus *Geospiza* being represented by more than one species. Because haptoglobin levels in blood can increase due to inflammation or trauma, a separate model was fitted with avian pox (*Avipoxvirus*: Poxviridae) infection state (pox) as a covariate. A bird was presumed infected when cutaneous nodules on the tarsus, digits and/or on tissue surrounding the beak and eyes were actively bleeding; a past infection was presumed from visual signs of old pox lesions like deformed or missing feet and digits (Parker et al. 2011).

## Results

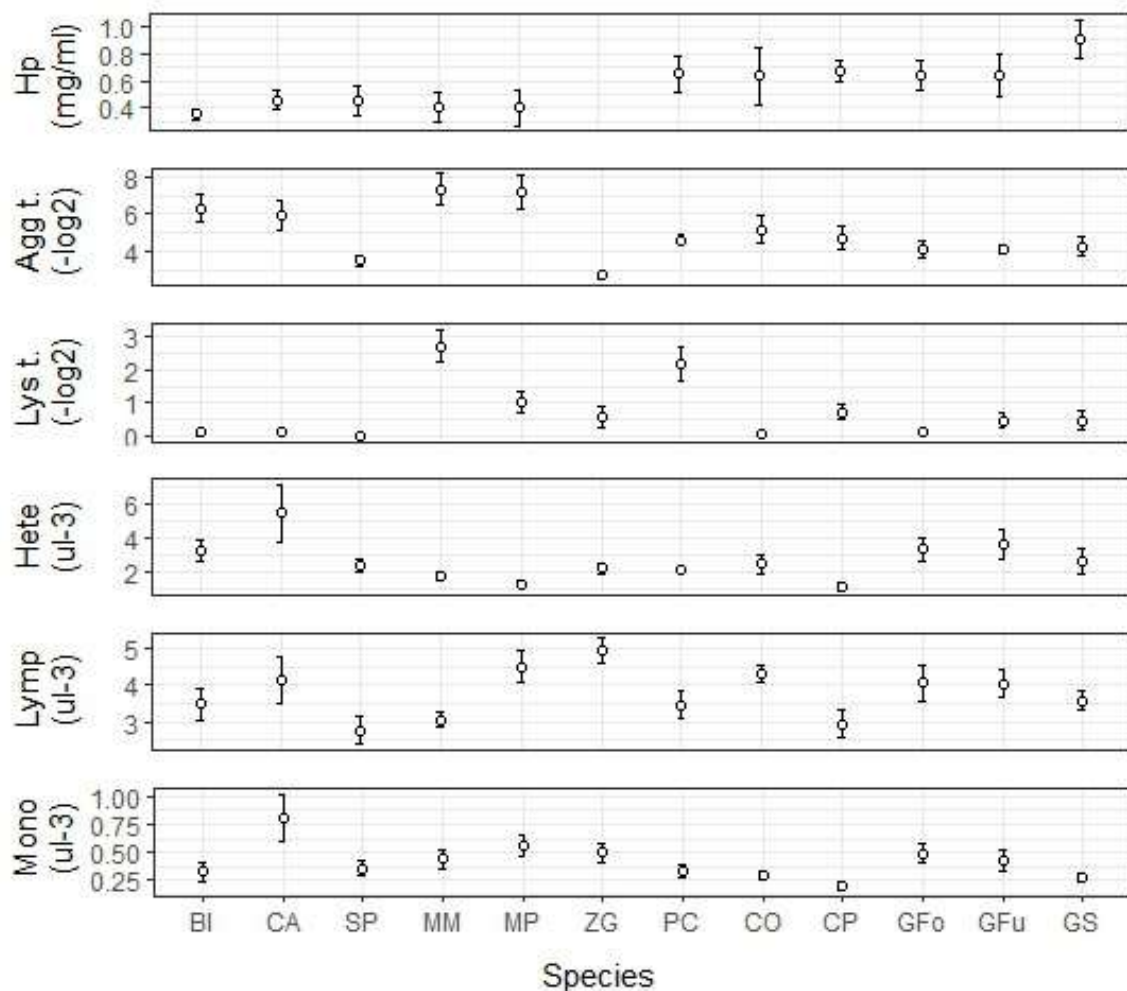
PGLS analyses revealed no significant relationship between time since arrival of a species to the archipelago and haptoglobin concentration, agglutination/lysis titers, and heterophil, lymphocyte, and monocyte concentrations. PGLS analyses with and without outliers provided consistent results. GLMM analyses found a positive association between haptoglobin and increasing time since arrival of a species, but no associations were found between time since arrival and the other five indices (Table 1, Figure 2).

**Table 1.** Model results for PGLS and GLMM analyses of the association between time since arrival of a species to the archipelago and each immune system index. The  $X^2$  statistic, degrees of freedom (df), significance value (p) and Holm-Bonferroni corrected significance value (p’) are provided for each index. Number of species per

test (no. species) and sample size (N) are also included where relevant. Significant values are shown in bold.

Immune Index	no. species	PGLS				GLMMS					
		$\chi^2$	df	p	p'	N	no. species	$\chi^2$	df	p	p'
Haptoglobin	10	6.181	1.000	<b>0.013</b>	0.142	125	11	10.351	1.000	<b>0.001</b>	<b>0.016</b>
Agglutination	11	0.002	1.000	0.966	1.000	187	12	1.499	1.000	0.221	1.000
Lysis	11	0.136	1.000	0.712	1.000	187	12	2.063	1.000	0.151	1.000
Heterophil	11	0.050	1.000	0.823	1.000	115	12	1.350	1.000	0.245	1.000
Lymphocyte	10	1.949	1.000	0.163	1.000	115	12	0.778	1.000	0.378	1.000
Monocyte	11	0.008	1.000	0.930	1.000	115	12	3.084	1.000	0.079	0.791

**Figure 2.** Association between time since arrival of a species to the archipelago and immune index by species.



Species are ordered by increasing time since arrival: *Bubulcus ibis* (BI), *Crotophaga ani* (CA), *Setophaga petechia* (SP), *Myarchus magnirostris* (MM), *Mimus parvulus* (MP), *Zenaida galapagoensis* (ZG), *Platyspiza crassirostris* (PC), *Cethidea olivacea* (CO), *Camarhychus parvulus* (CP), *Geospiza fortis* (GFo), *G. fuliginosa* (GFu) and *G. scandens* (GS). Immune indices abbreviations: Hp (haptoglobin conc.), Agg t. (agglutination titer), Lys t. (lysis titer), Hete (heterophil conc.), Lymp (lymphocyte conc.) and Mono (Monocyte conc.).

Pox infection had a marginally significant effect on haptoglobin concentration ( $X^2= 6.57$   $p=0.04$ ). Individuals with active pox lesions had the lowest levels of haptoglobin ( $t= -2.13$ ), followed by individuals with presumed old pox lesions ( $t=-1.62$ ), and lastly individuals with no signs of pox virus infection had the highest haptoglobin levels ( $t=3.11$ ). The relationship between haptoglobin and time since arrival to the archipelago remained significantly positive, even when the pox covariate was included in the model ( $X^2= 13.32$ ,  $p=0.0003$ ).

## **Discussion**

The limited diversity of parasites in most tropical archipelagos (Wikelski et al. 2004, Clark et al. 2014) suggests that host species on isolated islands have evolved under weak selection for immune responses (Jarvi et al. 2001, Van Riper and Scott 2001). We explored the immune system of birds in the Galapagos and compared species with different estimated times since arrival to the archipelago. We found no general trend between six indexes of the immune response to indicate that species that arrived to the islands more recently have overall stronger immune system function than species that have been there longer.

Similarly, Matson (2006) found no support for weakened immune system function in island birds compared to their continental close relatives. Matson suggested that different immune defense strategies may be favored for island species, due to tradeoffs between innate and acquired immunity. It has been further suggested that different defense strategies are optimal in parasite-poor versus parasite-rich environments due to tradeoffs between the antibody and cell-mediated immune response. In small ground finches (*Geospiza* sp.) in the Galapagos islands, as parasite prevalence and intensity increased with island size, the concentrations of natural

antibodies and the speed of specific antibody response also increased. However, the opposite pattern was shown for the strength of the cell-mediated immune response, suggesting a tradeoff, where a strong and rapid production of natural antibodies precedes cell-mediated immunity (Lindstrom et al. 2004).

In our study, we found an association only between increased haptoglobin concentration and the estimated time since arrival of species (Figure 2). The relationship is opposite to what might be expected if immune system function weakened with evolutionary time in isolation, but suggests that older species may have higher baseline or response levels of this acute phase protein. This result is consistent with Matson's (2006) study, in which island species showed significantly higher levels of haptoglobin than continental species. Supporting his suggestion of immune system reorganization, as at least one aspect of the innate defenses appears enhanced in island endemic species.

Because haptoglobin levels generally increase in response to infection (Cellier-Holzem et al. 2010, Ellis et al. 2014), our result could also mean that a larger proportion of individuals of the older species is responding to disease. However, haptoglobin was lower in individuals with active or presumed lesions caused by avian pox, a virus that commonly occurs in Galapagos endemic birds (Parker et al. 2011). Furthermore, our study included only individuals that tested negative for Haemosporidian parasites (*Plasmodium* and *Haemoproteus*), as part of a concurrent study (with the exception of *Z. galapagoensis*) (Jaramillo et al. 2017). Nonetheless, it must be noted that avian pox state was classified only through visual observation, and other diseases were not screened, thus we cannot rule out an effect of disease on haptoglobin levels.

Immunological indices in our study and Matson's (2006) have not provided patterns consistent with the hypothesis of weakened immune responses in isolated island avian life. However, an important caveat is that unaccounted biotic (e.g. ectoparasite loads) or abiotic factors could influence these immune indices. Furthermore, a number of events that have resulted in mass mortalities of wild and captive birds on islands involve introduced parasites to which birds had not been previously exposed (Warner 1968, Tompkins and Gleeson 2006, Bunbury et al. 2008). Major emergent events in the past few decades affected populations that are considered immunologically naïve due to lack of previous exposure to such parasites or pathogens (Daszak et al. 2000, Dobson and Foufopoulos 2001). Thus, further work is needed to understand the influence of island life on the evolution of the immune system of hosts.

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