January 2017

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**Recommended Citation**
Jaramillo, Maricruz; Rohrer, Sage; and Parker, Patricia, "From Galapagos Doves to Passerines: Spillover of Haemoproteus Multipigmentatus" (2017). *Biology Department Faculty Works*. 119.
DOI: [https://doi.org/10.1016/j.ijppaw.2017.07.001](https://doi.org/10.1016/j.ijppaw.2017.07.001)
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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 2254 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.

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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; Valkiunas, 2004; Valkiunas et al., 2006; Marzal et al., 2005; 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;
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*) (Valkiunas 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; Valkiunas 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*, re affirming the suspicion that rock pigeons were implicated in the introduction of the parasite to the archipelago. Rock pigeons were completely eradicated from the 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 speciﬁc nature of *H. multipigmentatus* in Galapagos doves (*Santiago-Alarcon et al., 2010*; Valkiunas 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*, re affirming 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 (*Valkiunas 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, 2014; Galen and Witt, 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 (Fig. 1). We used 2.5–6–m tall mist nets to capture passerines and other land birds and a 12 × 15 m drop net to capture cattle egrets. The number of birds captured divided by the sampling effort (nets × 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 1 h 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, dH2O solution (40× TNE2). Extracted DNA was inspected with an Epoch spectrophotometer (*BioTEK Instruments, Inc.*) for adequate OD260/O D280 values and DNA concentrations. For PCR-based molecular screening, a region of the parasite mitochondrial cytochrome b gene was amplified following Waldenström et al. (2004). One microliter of stock DNA was used in the initial reaction and 1 μL 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*, Karse 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 (×400) for 5 min followed by examination of 200 fields under high magnification (×1000) 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 (×1000). 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 10⁴ erythrocytes, were log transformed and compared with a two-sample t-test in R.

3. Results and discussion

We screened 2254 individuals from 22 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 (Fig. 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 (Valdúnas 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, \( F_s = 23.56, p = 0.002, \) Fig. 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 (Fig. 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 ± 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 birds found hummingbirds are reservoirs of generalist *Haemoproteus* parasites, which probably spill over to 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 (Valkiunas et al., 2014) that cannot be confirmed unless histological studies are performed (Dinhlop 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 (Valkiunas, 2011). This suggests that *Haemoproteus* spp. infections might be lethal to some non-adapted hosts at early stages.

**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 |
|------------------------------|-------------------------|-----------|--------|----------|-------|
| *Platyspiza crassirostris*    | Vegetarian Finch        | –         | 1      | –        | 1     |
| *Camarhynchus parvulus*       | Small tree finch        | 7         | 11     | –        | 18    |
| *Camarhynchus psittacula*     | Large tree finch        | 1 (1)     | –      | –        | 1 (1) |
| *Geospiza fortis*             | Medium ground finch     | 71 (1)    | 103 (7)| 2 (1)    | 126 (9)|
| *Geospiza fuliginosa*         | Small ground finch      | 7 (2)     | 140 (16)| 18 (4)  | 230 (32)|
| *Geospiza magnirostris*       | Large ground finch      | 2 (1)     | 12     | –        | 14 (1) |
| *Geospiza scandens*           | Cactus finch            | 1         | 2      | –        | 3     |
| *Myiarchus magnirostris*      | Galapagos flycatcher    | 6         | 22     | –        | 28    |
| *Mimus parvulus*              | Galapagos mockingbird   | 1 (1)     | 24 (6) | –        | 25 (7) |
| *Setophaga petechia*          | Yellow warbler          | 8 (1)     | 50 (7) | 3        | 65 (8) |
| *Zenaida galapagoensis*       | Galapagos dove          | 9 (9)     | 4 (4)  | 18 (18)  | 31 (31)|
| **Totals**                    |                         | 128 (26)  | 369 (40)| 41 (23)  | 538 (89)|

![Figure 2](image.png)

Fig. 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).
of parasite development (Valkiunas, 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 (0.7 ± 0.7, n = 6) than for negative G. fuliginosa (0.13 ± 0.06, n = 10, p = 0.01). For G. fortis the H/L-ratio was higher for positive individuals (0.74 ± 0.54, n = 3) than for negative (0.03 ± 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 58% 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 cases of stress or disease 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; Valkiunas and Izhova, 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 a larger number of sampling sites, 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 (r² = 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 columbiaform birds, histopathological examination of dead doves’ organs has revealed sufficient tissue damage to assume infection with Haemoproteus columbae as cause of mortality (Earlé 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 (r² = 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 (r² = –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, Microlychnia galapagoensis found in Galapagos Doves (Valkiunas et al., 2010) and Ornithoica vicina found in Galapagos passerines (Deen 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.

### Acknowledgements

This work was supported by the WildCare Institute of the Saint Louis Zoo, the Des Lee Collaborative Vision and the Whitney R. Harris World Ecology Center at the University of Missouri — Saint Louis. We thank Samoa Asigau, Courtney Pike, Jessica Griffard, Lauren Sage, Amy Wiedenfeld, Fredy Gaona, Macarena Parra, Mariana Vera, Lorena Rojas, Jeisson Zamudio, Jorge Luis Peña, Rob Jansen, Ana Morales, Anna Walentowitz, Jorge Castillo and Lillian Catenacci, for their valuable assistance at the field. Thanks to Cindy Rettke for her support and guidance in laboratory techniques and thanks to Iman Hussein, Pulcherie Guissou, Elham Mohammed, Barsha Baral, Arvon Clemens and Hannah Franko for their meticulous work in the laboratory. Many thanks to Dr. Gediminas Valkiunas who highlighted the importance of this work, motivated the production of the manuscript and provided knowledgeable advice. We thank Dr. Robert Ricklefs, Dr. Kathryn Huyvaert and the Parker Lab for comments on the manuscript. The Galapagos National Park Service provided the necessary permits and assistance and the Charles Darwin Research Station provided logistic support. This publication is contribution number 2162 of the Charles Darwin Foundation for the Galapagos Islands.
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