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The role of *Philornis downsi* in avian disease transmission in the Galápagos Islands

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DEDICATION

I lovingly dedicate this degree to my parents who always supported my educational aspirations--in memory of my father, Eugene (Gene) Pike, who also sparked and fueled my love and respect for nature and my mother, Glenda Pike. I also dedicate this thesis to my loving dog, Holly ('Jalapeño') Pike, my loyal companion for 18 years, who still gives me endless love even after many hours, days, and weeks in the lab away from her and kept me company during countless hours researching and writing from home and at the park.

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ABSTRACT

Philornis downsi is a dipteran fly species known to parasitize passerine birds in the Neotropics. *P. downsi* is speculated to be a recently introduced species in Galápagos Islands, so it is important to understand its effects on the archipelago's native and endemic avian species. Avian parasites and pathogens have been previously reported in the Galápagos, leading the Galápagos National Park and the Charles Darwin Foundation to focus efforts on the study and management of potential avian disease vectors. The main goal of this study was to determine whether *P. downsi* is a host for avian parasites and pathogens found in the Galápagos Islands. In Chapter I, I initially discuss introduced species as avian disease agents or vectors and highlight previous studies focused in Galápagos. Detailed background information on the life history and ecology of *P. downsi* is provided, followed by a review of the literature on insects as vectors of avian disease. In Chapter II, I report research conducted on *P. downsi*, assessing its capability of hosting avian parasites. Using molecular techniques, we tested *P. downsi* adults for haemosporidian parasites and *Trypanosoma*, as well as microfilarial nematodes, all parasites that have been described in Galápagos birds. We did not detect the presence of avian parasites of these genera, nor did we detect filarial nematodes; however, we did detect insect-specific trypanosomatids within *P. downsi* samples with a 0.90 overall prevalence. Our results suggest *P. downsi* is not a host of the avian parasites and pathogens for which I screened, indicating it is not a suitable vector; however, further research should be conducted. We recommend future studies to include testing of *P. downsi* larvae, an expanded geographical range for sample collection, and inclusion of other avian parasites and arboviruses.

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CHAPTER I

Background & Literature Review on *Philornis downsi* and Insects as Vectors of Disease

ABSTRACT

Introduced species may highly affect native species, acting as vectors of parasites or disease agents to native species, or may even parasitize native hosts directly. Avian species are highly susceptible to diseases transmitted by a wide range of vectors. In the Galápagos Islands, many avian parasites have been detected, including *Philornis downsi*, a fly parasitic to avian hosts. This chapter provides a detailed review of the life history and ecology of *Philornis downsi*, speculated to be an introduced species into Galápagos Islands. I discuss the widespread distribution of *P*. *downsi* within the Galápagos Archipelago, its life cycle, feeding behavior, parasitism rates and effects on avian hosts. I also review the current literature for studies on insects and their role in avian disease transmission. Examples of insects and the avian parasites they vector are discussed, along with avian parasite transmission modes. This chapter concludes with summary of current research on *P*. *downsi*, highlighting the gaps in the literature. Suggestions for further research include investigating *P*. *downsi* as a potential vector of avian parasites and arboviruses.

INTRODUCTION

Introduced species are common to islands (Simberloff, 2010), and some can have detrimental effects on these island ecosystems (Dvorak et al., 2012). These ecosystems may be affected at the individual, population, and community levels and native species can be greatly impacted by increased predation, competition, or disease (Simberloff, 2010). For instance, many island species lack behavioral defenses against new predators (Brock et al., 2014). In Guam, the introduced brown tree snake caused extirpations of many native bird

and reptile species due to predation (Fritts and Rodda, 1998). A comparative study by Campbell (1996) specifically tested the reactions of two gecko species in Guam to a predatory cue and found that the native species lacked defense reactions significantly more often than the species thought to be introduced. Likewise, Christmas Island experienced declining populations of the red land crab *Gecarcoidea natalis* in areas infested with the introduced yellow crazy ant *Anoplolepis gracilipes*. The red land crabs did not have defense behaviors against formic acid, emitted by the ants to stun the crabs, nor against swarms of these ants attacking the crabs' burrows (Dowd et al., 2003).

Native species in oceanic islands may also be the most vulnerable to introduced parasites and pathogens. Specifically, native species lack naturally acquired immunity to introduced diseases. Such was clearly the case in Hawaii, where introduced avian malarial parasites caused the extinctions of ~33% of the Honeycreeper species (Warner, 1968; Maclean, 2015). The Honeycreeper species living at higher elevations survived, as malaria did not reach these elevated areas (LaPointe et al., 2012). Higher susceptibility may also be attributed to dispersal limitations on islands, as many islands are not large enough and/or do not have a wide range of elevations to offer alternative refuge areas where native birds could avoid threats of parasites and their vectors (LaPointe et al., 2012).

The Galápagos Archipelago is a uniquely isolated system with many endemic avian species including the critically endangered Mangrove Finch (*Camarhynchus heliobates*) (IUCN, 2014). High levels of endemism and declining population sizes of some native avian species in Galápagos have increased the concern of extinction risk. While climate change and anthropogenic activities may be contributing factors to these drastic declines (Dvorak et al.,

2012), new parasites, viruses, and diseases may pose greater threats to these avian species than these aforementioned factors.

Many avian parasites have already been introduced to the Galápagos Islands. Concerningly, avian blood parasites of the genera *Plasmodium*, *Haemoproteus*, *Trypanosoma* along with filarial nematodes have been detected in avian species in Galápagos (e.g., Parker et al., 2006; Merkel et al., 2007; Levin et al., 2011, 2013). Infections with these parasites have a wide range of pathogenicity and virulence, from unnoticeable effects on their hosts to vomiting, anemia, behaviors resembling depression, and sometimes death (Greiner and Ritchie, 1994; LaPointe et al., 2012). While much research focuses on monitoring avian health in Galápagos, effects of these parasites on avian species are understudied. Currently, passerine health and population size on Galápagos are most noticeably affected by the avian pox virus (Kleindorfer and Dudaniec, 2006; Parker et al., 2006; Parker et al., 2011) and the avian parasite *Philornis downsi* (Diptera: Muscidae) (Fessl and Tebbich, 2002). This paper provides a review of the literature on *Philornis downsi* and insects as vectors of avian parasites.

PHILORNIS DOWNSI

Philornis downsi belongs to a genus that spans many countries in the Caribbean and Latin America. Out of 50 *Philornis* species (Couri et al., 2005), about half are known to specialize on and parasitize avian species (Fessl et al., 2001; Dudaniec et al., 2006), including *P*. *downsi*.

Spatial distribution

Studies have documented *P*. *downsi* in Sangre Grande (Dodge and Aitken, 1968) and St. Augustine, Trinidad (Couri, 1984) and in three sites in Brazil, including Angra dos Reis,

Rio de Janeiro (Mendonca and Couri, 1999), Dourados, Mato Grosso do Sul (Couri, 1999, as cited in Silvestri et al., 2011), and Nova Teutonia, Santa Catarina (Couri, 1984) and speculate *P*. *downsi* is a native species to these regions. Silvestri et al. (2011) reported *P*. *downsi* larvae in Parque Nacional Chaco, Argentina; however, it is unknown when *P*. *downsi* arrived there. While its presence in mainland Ecuador was unknown (Causton et al., 2013), Bulgarella et al. (2015) have recently reported *P*. *downsi* infesting passerine nests in Bosque Protector Cerro Blanco and Reserva Ecológica Loma Alta on mainland Ecuador.

P. *downsi* is believed to be a recently introduced species in Galápagos (Causton et al., 2006). Previous research, including a 30 year study begun in 1972 on two Darwin's finch species on Daphne Major, has not reported *P*. *downsi* parasitism in nests (Grant and Grant, 2002), suggesting a more recent arrival of *P*. *downsi* to the islands. In 1997, *P*. *downsi* was discovered in the nasal cavities of woodpecker finch nestlings on Santa Cruz Island (Fessl et al., 2001) in Galápagos; however, *P*. *downsi* specimens were found in a 1964 insect collection from Galápagos, indicating this species has a longer presence on the islands than previously thought (Causton et al., 2006). The method of introduction is unknown; however, speculations include anthropogenic activities like transportation of food, animals or other materials from Ecuador mainland to Galápagos (Causton et al., 2013).

P. *downsi* has a widespread distribution in the Galapagos Islands, with a presence on 13 of 15 islands surveyed (Wiedenfeld et al., 2007; Causton et al., 2013). Previous studies have found higher intensities of *P*. *downsi* parasitism in bird nests in habitats in highland areas (Wiedenfeld et al., 2007), compared to those in lowland areas. *P*. *downsi* is also thought to prefer higher altitude areas due to the moist environment and abundant resources (Dudaniec et al., 2007; Wiedenfeld et al., 2007; O'Connor et al., 2010a).

Life Cycle & Feeding Ecology

The life cycle of *P. downsi* includes three parasitic larval stages and one non-parasitic adult stage. Female flies lay their eggs in randomly chosen active passerine nests. Around day 2-3, the larvae hatch and first instars move into the nostril cavities of the nestlings, where they reside for 1-8 days. These larvae have specialized mouthparts, including mouth hooks and tooth-like projections, for their hematophagous lifestyle (Fessl et al., 2006b), in which they feed on the blood of nestling birds (Fessl and Tebbich, 2002). The larvae commonly target nasal cavities (Galligan and Kleindorfer, 2009), but may also feed on blood and tissue within wounds on the nestlings' bodies (Fessl et al., 2006b). Once mature, second instar larvae migrate to the nest base, where they stay during daylight hours. At night, however, the mature larvae are ectoparasitic, returning to nestlings to obtain blood meals throughout the night. After about seven days in the nest base, the mature larvae pupate. The pupae remain in the base of the nest for approximately 14 days, after which they exit the nest as adult flies (Fessl et al., 2006b; Causton et al., 2013). *P. downsi* adults are only known to feed on decaying organic matter (Fessl et al., 2001); however, other food sources may exist. In general, little is currently known about the life history of this species, especially at the adult stage (Dudaniec and Kleindorfer, 2006; Dudaniec et al., 2010).

Rates and effects of P. downsi parasitism

As a generalist, *P. downsi* parasitizes passerine nests in the Galapagos Islands. Its hosts include 15 endemic, two native but widespread, and one introduced species (Causton et al., 2013). In a study by Fessl and Tebbich (2002), *P. downsi* larvae were found in 97% of active finch nests on Santa Cruz Island. Similarly, Koop and colleagues reported 90% of medium ground finch (*Geospiza fortis*) nests examined (43/48) on Santa Cruz Island had *P.*

downsi parasitism (2011). In a prior study on *Philornis* parasitism in Puerto Rico, Arendt (1985) found over 95% of pearly-eyed thrasher (*Margarops fuscatus*) nests infested with *Philornis deceptivus*, comparable to the abovementioned studies on *P. downsi*. Furthermore, averages of 28-48 of *P. downsi* larvae may infest each nest (Kleindorfer et al., 2014), with a maximum of 182 larvae detected in one nest (Fessl and Tebbich, 2002).

Parasite intensity of *P. downsi* on avian hosts varies depending on different factors. Dudaniec and Kleindorfer (2006) showed parasite intensity is significantly higher per nestling in smaller broods. Huber (2008) found the timing of egg laying and hatching to be a significant factor in parasitism intensity. Higher levels of *P. downsi* parasitism were recorded in early breeding season nests compared to nests laid later in the season.

Studies have reported detrimental effects on birds parasitized by *P. downsi*. Galligan and Kleindorfer (2009) reported deformities of the nasal cavities and beaks of 36% of small ground finches (*Geospiza fuliginosa*) surveyed on Santa Cruz Island. Fessl et al. (2006a) also mentioned enlarged nasal cavities while examining the small ground finch (*G. fuliginosa*) and the medium ground finch (*G. fortis*). This study also found extensive (up to 55%) blood loss and decreased hemoglobin (Hb) levels in these parasitized birds, supporting evidence from a study on *G. fuliginosa*, which also found lower hemoglobin levels in nestlings compared to adult birds (Dudaniec et al., 2006). Fessl et al. (2006a) also recorded lower levels of mass gains in parasitized nestlings. In contrast, Huber (2008) found no significant differences in *G. fortis* nestling size or growth rate when comparing nestlings with and without *P. downsi* larvae. Similarly, a study on adult *G. fuliginosa* did not detect significant differences in body size or condition in deformed (indicating previous parasitism) birds compared to normal birds sampled (Galligan and Kleindorfer, 2009).

Parasitism by *P*. *downsi* can greatly affect fledging success and reproductive fitness of many avian species. Many studies have reported decreased fledging success rates. For example, Dudaniec et al. (2006) showed decreased fledging success as mean parasite intensity per nestling increased. Fessl et al. (2006a) reported a 33% fledging rate of *G*. *fuliginosa* and *G*. *fortis* nestlings in untreated nests examined, while Koop et al. (2011) found only 4% (3/67) of *G*. *fortis* nestlings fledged. In addition, Huber (2008) found higher nestling mortality (≤ 100%) in parasitized nests examined over a three-year study.

Furthermore, declines in many of *P*. *downsi*'s host species have been recorded, mainly by studies conducted on Santa Cruz Island. While estimating land bird population sizes, Dvorak et al. (2012) found the woodpecker finch (*Camarhyncus pallidus*) and the warbler finch (*Certhidea olivacea*) had the greatest declines in population sizes. These findings correlate with data showing these two species had the most intense *P*. *downsi* parasitism levels (Dudaniec et al., 2007). Alarmingly, *P*. *downsi* parasitism has also pushed the mangrove finch (*C*. *heliobates*), which resides only on Isabela Island, to the brink of extinction (Fessl et al., 2010; IUCN, 2014).

INSECTS AS VECTORS OF AVIAN PARASITES

Natural behaviors of insects, including blood-feeding from birds, provide an ideal mode of transmission for parasites to susceptible hosts. In most insects, hematophagy is considered a rare behavior (Lukashevich and Mostovski, 2003); however, the insect order Diptera, specifically, contains many species with biting and sucking mouthparts (Kondratieff, 2005), well adapted for blood feeding, a known transmission method of many avian parasites. Previous studies have shown numerous dipteran species are capable vectors of avian parasites and viruses, including species of mosquitoes (Diptera: Culicidae). Studies

have shown that *Culex* mosquitoes are vectors of *Plasmodium relictum*, an avian malarial parasite, to Hawaiian bird hosts (LaPointe et al., 2005). Many avian viruses including the St. Louis encephalitis virus, Eastern equine encephalitis, and Western equine encephalomyelitis are also vectored by *Culex* and *Coquillitidia* mosquitoes (Eldridge, 2005). In addition, black flies (Diptera: Simuliidae) transmit *Leucocytozoon* and *Trypanosoma* parasites to many avian species (Adler, 2005). Also, a biting midge, *Culicoides circumscriptus* (Diptera: Ceratopogonidae), is suspected to be a vector of *Haemoproteus* parasites to two passerine species (Ferraguti et al., 2013). Other biting midges are known vectors of many viruses (including the alphavirus and bunyavirus), filarial nematodes, and *Haemoproteus* parasites to avian hosts as well (Borkent, 2005). Additionally, Baker (1956) showed the hippoboscid flies (*Ornithomyia avicularia)* (Diptera: Hippoboscidae), transmit *Trypanosoma avium* to birds. More recent studies suggest hippoboscid flies also vector *Haemoproteus multipigmentatus* and *H. iwa* to Galápagos doves (*Zenaida galapagoensis*) and frigatebirds (*Fregata*), respectively (Valkiūnas et al., 2010; Levin et al., 2011). Furthermore, *Musca domestica* (Diptera: Muscidae), the house fly, is a suspected vector of *Yersenia pseudotuberculosis*, a parasite in turkeys, as a study by Zurek et al. (2001) detected these parasites in the flies.

Avian parasites have a range of transmission modes from insect vectors to hosts. This is especially the case for avian trypanosome transmission. For instance, insects can potentially transmit trypanosome parasites via regurgitation by the insect vector into the blood and tissue of avian species during feeding via biting/blood sucking (Volf et al., 2004). One study by Van Dyken et al. (2006) detected avian trypanosomes within mosquito species

Culex pipiens and *C. tarsalis*, suggesting possible transmission of avian trypanosomes through biting.

Ingestion of insect vectors containing parasites has also been shown to be a route of transmission of parasites in some taxa. For example, sand flies (Genus: *Lutzomyia*) have been found to transmit *Trypanosoma* parasites to fence lizards (*Sceloporus occidentis*) and forest geckos (*Thecadactylus rapicaudus*) by ingestion (Olsen, 1974). In addition, in experimental studies avian trypanosomes were shown to be transmitted to canaries (*Serinus canaria*) after ingestion of infected *Culex* mosquitoes (Votýpka et al., 2012) and black flies (*Eusimulium latipes*) (Votýpka and Svobodová, 2004). Adler (2005) also suggested ingestion of black fly vectors as a mode of transmission for *Trypansoma corvi* to European birds. While the adult *P. downsi* flies do not take blood meals (Fessl et al., 2001), there is evidence of birds ingesting *P. downsi* larvae and the adult flies (O'Connor et al., 2010b), indicating the possibility of transmission of trypanosomes from potentially infected *P. downsi* flies to avian host species.

CONCLUSION AND FURTHER RESEARCH

Overall, there is a growing body of literature on *P. downsi*, mostly focused on its general ecology and parasitic effects on avian hosts. More research is needed to determine the extent of *P. downsi*'s distribution, including further surveys in countries where this species has previously been detected and in countries not yet surveyed. Research studies to measure and monitor *P. downsi*'s effects on avian species should also be conducted. In addition, while *P. downsi* larvae are not thought to parasitize adult birds, a study by Arendt (1985) reported *Philornis deceptivus* larvae parasitizing adult pearly-eyed thrashers (*M. fuscatus*) in Puerto Rico. Thus, it may be possible for adult birds to be parasitized in the

future by *P*. *downsi*, so more monitoring should also focus on checking adult birds for these parasites.

While numerous studies have been conducted on avian hosts, their parasites, and vectors, many gaps in knowledge still exist. As noted, much research on vectors of avian parasites has focused on dipteran insects; however, there seems to be a bias toward studying mosquitoes and black flies. Studies are lacking in investigating the presence of transmissible parasites and pathogens in *Philornis* species, including *P*. *downsi*. Generally, *Philornis* species should be investigated as potential avian disease vectors. In addition, the close relationship between *P*. *downsi* larvae and nestling birds could play a role in disease transmission. Given *P*. *downsi*'s life history and parasitism on nestling birds, it is necessary to determine if *P*. *downsi* is a competent vector for any avian pathogens. Further research may include studies testing *P*. *downsi* for transmissible avian disease agents that require a vector, such as malarial parasites, trypanosomes, and viruses like West Nile virus.

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CHAPTER II

Philornis downsi as an avian disease vector in Galápagos Islands

ABSTRACT

There has been scant research on the life history and ecology of *Philornis downsi*, a fly and avian parasite, including its potential role in disease transmission. *P. downsi* has colonized the Galápagos Islands, where infectious disease agents including blood parasites and other pathogens have been reported in avian species. Given the close relationship *P. downsi* has with passerine birds on the Galápagos Islands, our study investigates *P. downsi* as an avian disease vector. *P. downsi* adult flies were caught using McPhail traps on Santa Cruz and Isabela, Galápagos Islands. Using polymerase chain reaction (PCR) molecular techniques, we screened *P. downsi* adults for the presence of avian blood parasites and pathogens including genera *Plasmodium*, *Haemoproteus*, and *Trypanosoma*. We also screened for microfilarial nematodes, groups of parasites that have been described in Galápagos birds. PCR testing targeted regions of the parasites' mitochondrial cytochrome b and COI genes, as well as the SSU rRNA gene. Samples positive for parasites in the Trypanosomatidae family were sequenced for parasite identification. We did not detect the presence of avian blood parasites and pathogens in *P. downsi* samples; however, our data do provide evidence of insect-specific trypanosomatids infecting *P. downsi* samples, with an overall prevalence of 0.90. Our results suggest that *P. downsi* is not a competent host for the avian parasites we tested for and may not play a role in vectoring these parasites. We recommend further studies on other developmental stages, an expanded geographical range for sample collection, and testing for other avian parasites and arboviruses.

Keywords: *Philornis downsi*, Galápagos, disease transmission, avian disease

INTRODUCTION

Globally, avian parasites and disease are recognized threats to avian health (Friend et al., 2001; Parker et al., 2006). Novel parasites and diseases may especially threaten avian species in island systems, which may provide naïve environments ideal for colonization by introduced species. Avian malaria, caused by the introduced parasite *Plasmodium relictum*, has been implicated in the extinction of many endemic honeycreeper species in the Hawaiian Archipelago (Maclean, 2015). This raises the concern for another unique island system, the Galápagos Archipelago, which harbors many native and endemic avian species. Prior studies have already reported the presence of avian blood parasites and filarial nematodes in Galápagos (e.g., Parker et al., 2006; Merkel et al., 2007; Levin et al., 2011, 2013); however, the effects of these parasites on avian populations have not been extensively documented. Currently, declines in many Galápagos passerine bird populations, including the critically endangered Mangrove Finch (*Camarhynchus heliobates*), are attributed to another parasite, *Philornis downsi*, a fly speculated to be an introduced species (Fessl et al., 2010).

During its larval stages, *P*. *downsi* parasitizes passerine nestlings, feeding on their blood and tissue (Fessl et al., 2002, 2006b). *P*. *downsi* parasitism on avian hosts was first documented in Galápagos in 1997 on Santa Cruz (Fessl et al., 2001), yet the date and mode arrival of *P*. *downsi* to this archipelago are unknown, despite *P*. *downsi* presence in insect collections dating back to 1964 (Causton et al., 2006). Given *P*. *downsi*'s widespread distribution (Wiedenfeld et al., 2007; Causton et al., 2013) and its detrimental effects on nestlings (see: Dudaniec et al., 2006; Fessl et al., 2006a; Koop et al., 2011), Causton and colleagues published a management plan for the control of *P*. *downsi* in the Galápagos (2013). This plan presented questions and information on *P*. *downsi*, including topics such as

mating sites, reproductive biology, and dispersal ability, and the question "Is *P*. *downsi* a vector of disease?" (Causton et al., 2013). While the literature indicates that some *Philornis* species can transmit arboviruses to avian species (Aitken et al., 1958), this capability is currently unknown for *P*. *downsi* (Causton et al., 2013). Since *P*. *downsi* larvae are known to parasitize passerine hosts (Fessl et al., 2002, 2006b), it is important to assess their risk of avian disease transmission via *P*. *downsi*.

In this study, we investigated the presence of transmissible avian parasites and pathogens in adult *P*. *downsi* flies in Galápagos. Through collaborative efforts with the Charles Darwin Foundation and the Galápagos National Park, *P*. *downsi* adults were collected at sites along an elevational gradient on Santa Cruz and at breeding sites of the critically endangered mangrove finch on Isabela. Using molecular techniques, we tested *P*. *downsi* adults for parasites in the genera *Haemoproteus*, *Plasmodium*, and *Trypanosoma*, as well as for microfilarial nematodes. Transmission methods of these parasites include regurgitation of parasites by a biting vector into wounds of a host (Volf et al., 2004), ingestion of an insect vector by a host (Votýpka et al., 2012), and other speculated methods. Given the lack of evidence of *P*. *downsi* adult flies taking blood meals from birds (Fessl et al., 2001), we predicted that no avian parasites would be detected. However, ingestion of *P*. *downsi* adults by Galápagos birds has been documented (O'Connor et al., 2010), which could provide a method of transmission.

Results of this study will inform planning initiatives for management and control of *P*. *downsi* and disease vectors in Galápagos. The conservation of native and endemic avian species in this archipelago is very important and identifying vectors of avian diseases present on these islands is pertinent for successful conservation.

MATERIALS AND METHODS

Study site

Sampling took place in the Galapagos Islands on Santa Cruz and Isabela, two inhabited islands (Figure I). Collection occurred at low (15-41 meters (m)), mid (209-216 m), and high (589-616 m) elevations on Santa Cruz: at El Barranco (0˚44'34.1"S, 90˚18'10.4"W), Los Guayabillos (0˚41'68.7"S, 90˚20'78.6"W), and Los Gemelos (0˚ 37'82.0"S, 90˚23'44.4"W) sites (Figure II). On Isabela, collection sites were located at the mangroves at Playa Tortuga Negra (0°14'32.09"S, 91°23'10.97"W) and the lava area (0°14'46.69"S, 91°23'5.64"W) near Playa Tortuga Negra (Figure III). Collection of the specimens took place between March 23 and July 26, 2013. In total, 390 samples were received from these sites. Samples were pools of 1-7 flies, for a total of 923 flies. Total number of samples collected from each site varied, with 217 from El Barranco, 153 from Los Gemelos, and 2 from Los Guayabillos on Santa Cruz. On Isabela, 14 samples were collected from the mangrove site and 4 samples from the lava site.

Sample collection

Collaborators at the Charles Darwin Research Station collected adult *P. downsi* samples. McPhail traps were used to trap flies using fresh papaya juice as an attractant in the traps. Thirty traps were placed at each of the three collection sites on Santa Cruz and the mangrove site on Isabela. In addition, 24 traps were placed at the lava collection site on Isabela. On Santa Cruz, traps were hung in trees three to four meters above ground level at each trapping site and *P. downsi* adult flies were collected every three to five days. On Isabela, traps were hung at five, seven, and ten meters at both sites and *P. downsi* adult flies were collected within ten days of trapping. *P. downsi* samples from each trap were stored in

1.5 ml Eppendorf tubes containing 70% ethanol. In August 2013, samples were imported into the United States for genetic testing.

Deoxyribonucleic acid (DNA) extraction

Samples of adult *P. downsi* flies were analyzed in Dr. Patricia Parker's laboratory at the University of Missouri-St. Louis. Each sample was dried out for 12-16 hours (overnight) to ensure all ethanol evaporated from the flies before extraction. Some samples with 5-7 flies contained too much fly tissue for proper extraction, so these were separated into two samples before DNA extraction, yielding a final count of 401 samples for extraction. Next, DNA was extracted from each sample (containing 1-6 flies) using the QIAGEN DNeasy Blood and Tissue Kit. After extraction, samples were individually read on a Nanodrop spectrophotometer at wavelengths of 260 and 280 to determine the concentration of DNA. Samples with 80 ng/ µl DNA or less were not used for further testing. Following this, DNA samples were run on a small agarose gel to test for DNA degradation. Heavily degraded samples were excluded from testing. In total, 297 samples showed little degradation and were used for PCR testing. This included 165 from El Barranco, 110 from Los Gemelos, 2 from Los Guayabillos, 17 from the mangrove site, and 3 from the lava site. We tested a total of 295 samples for haemosporidians, 82 samples for filarids, and 297 samples for initial trypanosomatid testing with Sehgal et al. (2001) primers. For further trypanosomatid testing, 221 samples were tested with Valkiūnas et al. (2011) primers and 95 samples were tested with Votýpka et al. (2012) primers. Prevalence data reported are based on pooled samples.

Molecular testing

Molecular screenings were conducted using both single and nested polymerase chain reaction (PCR) tests to determine the prevalence of haemosporidian blood parasites,

trypanosomes, and microfilarial nematodes that specifically infect avian species. The nested PCR is a reliable method for parasite detection and has higher specificity for parasite identification than a single PCR (Sehgal et al., 2001; Waldenström et al., 2004).

Haemosporidian screening: To test for haemosporidian blood parasites in the genera *Plasmodium* and *Haemoproteus*, we conducted a nested PCR test that amplifies regions of the mitochondrial cytochrome b gene in the parasite, a gene commonly targeted for detection of these parasites (Waldenström et al., 2004; Levin et al., 2012; Carlson et al., 2013) (Table I). For the outer reaction, a 25 µl mix was created using the following reagents with their initial stock concentrations: 2.5 µl 10X Ex Taq ™ Buffer (TaKaRa), 2.0 µl deoxynucleotide triphosphates (dNTPs) (10 mM), 1.75 µl $MgCl_2$ (25 mM), 1.0 µl of each primer (10 uM), 0.125 µl TaKaRa Ex Taq DNA Polymerase, 15.625 µl nuclease-free water (ddH_2O), and 1.0 µl of DNA sample. For the inner reaction, a 25 µl mix was prepared again using the same reagents, volumes, and concentrations as the initial PCR, except using the inner primers and adding 1 µl of the initial PCR amplicon in place of the DNA. This second PCR targeted a 524 base pair fragment, nested within the amplified initial PCR product (Waldenström et al., 2004). Positive controls used were Galápagos penguin (*Spheniscus mendiculus*) samples that have tested positive reliably.

Filarid screening: A PCR test for microfilarial nematodes was also conducted on a subset of samples that represented each collection site using primers from Casiraghi et al. (2001) (Table I). The reagents for the PCR mix were the same as in Merkel et al. (2007), except only using half the amount cited for each component, and 1 µl DNA mixed with 1.5 µl ddH_2O instead of 2.5 µl DNA. This PCR amplifies a 688 base pair fragment of the mitochondrial cytochrome c oxidase subunit I (COI) gene. Targeting regions within this

gene can be useful for identifying nematode species (Derycke et al., 2010). Reliable positive controls used were from flightless cormorant (*Phalacrocorax harrisi*) and Galápagos penguin (*S. mendiculus*) samples. Resulting amplicons from both haemosporidian and filarid testing were run on a 1.5% agarose gel for scoring.

Trypanosoma screening: Two nested PCR reactions and a single PCR reaction were used to detect and identify trypanosome parasites (Table I). These PCR tests amplify fragments in the conserved region of the small subunit (SSU) ribosomal ribonucleic acid (rRNA) gene of the parasite, a gene commonly targeted for trypanosome detection and identification (Sehgal et al., 2001; Valkiūnas et al., 2011; Votýpka et al., 2012; Nzelu et al., 2014).

The first PCR conducted on all samples was a nested reaction that amplified a 326 base pair fragment (Sehgal et al., 2001). This nested PCR has been previously used to detect trypanosomes in avian and mosquito species (Sehgal et al., 2001; Van Dyken et al., 2006). This test included bovine serum albumin (BSA) in the PCR mix. Positive controls used were from mosquito and great frigatebird (*Fregata minor*) samples that have tested positive reliably. After a PCR test was completed, the amplicons were run on a 1.5 % agarose gel, and samples with bands at 326 base pairs were scored positive for trypanosomatids.

The second nested PCR was only used to test samples that scored positive for trypanosomatids with the first nested PCR that used primers from Sehgal et al. (2001). This second nested PCR targets a 770 base pair fragment (Valkiūnas et al., 2011). Two positive controls used initially were samples from a yellow-breasted chat (*Icteria virens*) and a White-eyed Vireo (*Vireo griseus*), followed by using *P. downsi* samples, all of which reliably tested

positive. The resulting PCR amplicons were run on a 2% agarose gel and bands at 770 base pairs were scored as positive.

The final PCR test for trypanosomes was conducted used primers from Votýpka et al. (2012) using a small subset of samples that scored positive for trypanosomatids using the Valkiūnas et al. (2011) primer sets. This PCR test was a single reaction that targets a 1300-1400 base pair fragment (Votýpka et al., 2012). These amplicons were also run on a 2% agarose gel and positive amplicons with bright bands were saved for sequencing.

Reagents and mix amounts for trypanosome PCR tests are listed in Table II. In addition, negative controls were included in each PCR reaction, using ddH_2O in place of genomic DNA.

Sequencing

We attempted to identify the species of the trypanosomatid parasites detected in *P. downsi* samples by sequencing positive samples. PCR amplicons scored as positive for trypanosomatids from Sehgal et al. (2001) and Votýpka et al. (2012) primer sets were purified using an Exonuclease I (Exo I) and Antarctic Phosphatase reaction (New England BioLabs Inc., Ipswich, Massachusetts). Using Valkiūnas et al. (2011) primers, the PCR yielded results with considerable non-specific banding. Even after optimization trials, the PCR yielded mostly samples with positive bands unusable for sequencing. Positive amplicons from Valkiūnas et al. (2011) primers were run on a 0.8% agarose gel, cut out and trimmed, and subsequently purified using a NucleoSpin Gel and PCR Clean-up kit (Machery-Nagel). Next, primers used for each PCR test were then used in subsequent sequencing reactions with the Big Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California). PCR sequencing products were cleaned up using an

ethanol/ ethylenediaminetetraacetic acid (EDTA)/ sodium acetate precipitation. Products resuspended in Hi-Di™ Formamide (Applied Biosystems) were sequenced on an ABI 3130xl genetic analyzer (Applied Biosystems) at the University of Missouri-St. Louis. Sequences were assembled and edited in Seqman Pro 8.0.2 (DNAStar, Lasergene). Any sequence with double peaks was omitted from further analysis. Next, sequencing results were entered into National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) to identify sequence matches and top matches were recorded.

Initially, a subset of samples testing positive by PCR using Sehgal et al. (2001) primers were sequenced. The resulting sequences were short in length and yielded many 100% identity matches with sequences in GenBank, spanning multiple genera. Primers from Valkiūnas et al. (2011) and Votýpka et al. (2012) were used for obtaining longer sequences for more specific identification. Using the gel-extracted amplicons from the nested PCR with primers from Valkiūnas et al. (2011), we obtained sequencing data for five samples; however, we are most confident in the blast matches of the one sample that has double-stranded sequence data. Amplicons from PCR tests using R-221 and Medlin B primers (Votýpka et al., 2012) were also sequenced. We are more confident in the results from this primer set as we have double stranded sequence data for all ten sequences blasted.

Statistical Analysis

The Chi-squared test with Yates' continuity correction was applied to test for differences in infection counts between the islands of Santa Cruz and Isabela and between El Barranco and Los Gemelos collection sites. Fisher exact tests were run for other pairwise comparisons between sites, except for the Los Guayabillos and Lava collection sites, due to small sample sizes.

A binary logistic regression was performed in R to determine the effect of collection month on infection prevalence. This test only included 274 samples from Santa Cruz with specified collection dates. Since our dependent variable, infection presence/absence, was binary, dummy variables were assigned using values of 1 = infected sample and 0 = uninfected sample.

Phylogenetic analysis

We used MEGA 6 for estimating a phylogenetic tree. Only sequences acquired from infected *P. downsi* samples using the Votýpka et al. (2012) primer set were included in this analysis. We created an alignment in MEGA which included our most reliable sequences and known sequences of insect or bird infecting species from the Trypanosomatidae family, obtained from GenBank. We aligned sequences using Clustal W and manually trimmed and edited the alignment. A model of best fit for DNA was determined and a maximum likelihood tree was constructed using the K2+G model (Kimura, 1980) with 1000 bootstrap replicates in MEGA 6 (Tamura et al., 2013).

RESULTS

Molecular testing

Of 295 pooled samples tested for *Plasmodium* and *Haemoproteus* malarial parasites, all had negative PCR results. In addition, a subset of 82 samples representing each collection site was tested for microfilarial nematodes, yielding a prevalence of 0.0 for all sites.

Trypanosome screening: Three separate sets of primers were used for PCR tests to broadly screen for trypanosomatids, including *Trypanosoma* species. Each primer set yielded different values for prevalence.

Out of 297 pooled samples tested using primers S-762 and S-763 (Sehgal et al., 2001), total trypanosomatid prevalence was 0.90. Parasites were present in pools from all months of collection (March-July 2013). Infection was widespread, with positive detection from samples of all collection sites (Figure IV). For collection sites on Santa Cruz, prevalence was 0.84 for El Barranco, 0.99 for Los Gemelos, and 1.0 for Los Guayabillos. Trypanosomatid prevalence on Isabela was 0.82 for the mangrove site and 1.0 for the lava site (Figure V). From the 221 samples we tested, 152 samples were positive by PCR using primers from Valkiūnas et al. (2011), with a prevalence of ~0.69. Of the 95 samples tested using primers from Votýpka et al. (2012), 40 were positive, yielding a prevalence of 0.42.

All sequences individually blasted to compare with known reference sequences in GenBank most closely matched with *Crithidia*, *Blastocrithidia*, and *Leptomonas* (Table III), all genera of monoxenous trypanosomatids, indicating the parasites detected only require one host for their life cycle. Our sequences from the Votýpka et al. (2012) primers did not have 100% identity with matches in BLAST; however, the one sequence we are confident in from the Valkiūnas et al. (2011) primers did have 100% identity with two species of *Crithidia*. Importantly, our sequencing results highly suggest that these are insect-specific parasites that require *P. downsi* as their only host, which rules out avian species as suitable hosts. In addition, using a Yates' Chi-squared test, prevalence of infected samples collected from Los Gemelos was significantly higher than El Barranco ($X^2 = 15.589$, df = 1, $p < 0.001$). Applying Fisher's exact test indicated parasite infection prevalence for Los Gemelos was significantly ($p = 0.00747$) higher than the prevalence for the mangrove site, with high power (0.976). No other significant differences between sites or between islands were found. The binary logistic regression indicated month of collection had an overall significant (deviance =

15.255, d.f. = 3, $p < 0.001$) effect on infection prevalence in samples collected. April and May had the highest and lowest prevalence, respectively.

Phylogenetic Analysis

Our phylogenetic tree (Figure VI) indicates our trypanosomatid parasite sequences group with insect-specific trypanosomatid species, supported with high bootstrap values. Furthermore, the branching shows distinct separation between the *Trypanosoma* clade (avian trypanosomes) and the insect-specific clades.

DISCUSSION

Our test results indicate an absence of avian blood parasites and pathogens in *P. downsi* adults. This suggests that *P. downsi* may not be capable of hosting avian parasites for transmission and may not play a role in vectoring avian blood parasites of the genera *Plasmodium*, *Haemoproteus*, and *Trypanosoma*, nor vector parasitic nematodes in the Galápagos Islands. While we did not identify the newly discovered trypanosomatids to be avian parasites, *P. downsi* may still be a competent host for other avian parasites and pathogens, such as *Avipoxvirus*, already detected in Galápagos (Parker et al., 2011). Additionally, *P. downsi* may act as a dead-end host if infected with avian parasites. Given our results, we do not believe *P. downsi* is a current host of the parasites we screened for; however, if new avian parasites, pathogens, or viruses are introduced into the Galápagos Archipelago, we cannot rule out that *P. downsi* may not be a capable host and vector.

This is the first known report of parasites infecting the dipteran species *P. downsi*. Due to our concern for transmission of avian trypanosomes and *P. downsi*'s potential role, we examined the sequences and the genera that they closely match. The Trypanosomatidae family is comprised of two dixenous genera, *Leishmania* and *Trypanosoma*, the latter of

which includes avian trypanosome species (Zídková et al., 2012). Dixenous species require multiple host species (e.g. an insect host and avian host) to complete their life cycles. However, the Trypanosomatidae family also includes many monoxenous genera, meaning they only infect one host, most commonly an insect (Maslov et al., 2013). In our study, BLAST sequence matches and our phylogenetic hypothesis suggest trypanosomatids detected in *P*. *downsi* are most closely related to species in the insect-specific genera *Crithidia*, *Leptomonas*, and *Blastocrithidia*, indicating these parasites do not currently pose a threat to the avian species in the Galápagos Islands.

These trypanosomatids might be transmitted vertically (from a parent to offspring) or horizontally (from host to host). If transovarial vertical transmission is taking place, it seems that *P*. *downsi* would be initially infected during the egg and larval stages; however, if transovum vertical transmission is responsible for infecting individuals, then it may be possible for *P*. *downsi* adults to acquire these parasites via ingestion of eggshells with parasites present on them, as shown in experimental studies on transovum trypanosomatid transmission in the milkweed bug (*Oncopeltus fasciatus*) (Dias et al., 2014). Studies have also reported horizontal transmission of trypanosomatids in insects, including *Triatoma infestans* (Schaub and Jensen, 1990) and *Bombus* bumblebees (Erler et al., 2012) via infected insect fecal matter. *P*. *downsi* may be acquiring parasites via this transmission mode. This also raises the concern of infection of other insect species in Galápagos, as some may also feed on organic matter contaminated with *P*. *downsi* feces, possibly containing trypanosomatids. These parasites may not be host specific, indicating *P*. *downsi* would not be the only host species; however, if these parasites are currently host specific, there is a risk that host switching from *P*. *downsi* to other insect species in Galápagos may occur in the

future. It does not seem feasible that these parasites would be capable of switching to avian hosts.

This study provides further evidence that the primer sets used in Sehgal et al. (2001), originally intended for testing bird blood samples, work reliably in some insect species including *Culex* mosquitoes (Van Dyken et al., 2006) and *P*. *downsi* for trypanosomatid detection. In this study, using the same primer set and optimized conditions derived from Sehgal et al. (2001), we had positive amplification for trypanosomatids in 266 out of 297 *P*. *downsi* samples tested. Our work also coincides with previous studies that found trypanosomatids, based only on gene sequences (e.g., in terrestrial leeches: Hamilton et al., 2005; in *Culex* mosquitoes: Van Dyken et al., 2006).

Comparing the sensitivity of primer sets for trypanosomatid detection yielded discrepancies in prevalence results. The tests with a larger target fragment size did not yield as high prevalence values compared to the initial tests using the primers that targeted a 326 bp fragment. Furthermore, relying on PCR methods for detection of parasites and pathogens has its own limitations, including occasional false negatives. This indicates our testing most likely missed amplification of trypanosomatids in some samples. Nevertheless, we are confident in the positive results of our PCR-screened samples. In addition, the initial primer sets used for trypanosome testing yielded small fragment sizes, which were not useful for parasite identification using sequence data. Therefore, we suggest a closer examination of the literature and reference databases for the most appropriate primer sets, given a specific aim, and more standardized methods for identifying species within Trypanosomatidae. Additionally, we acknowledge the limit of molecular techniques and suggest use of *P*. *downsi* salivary gland and midgut slides for increased confidence in parasite identification.

This study focused research efforts on *P*. *downsi* adult flies collected from Santa Cruz and Isabela islands, two of at least 13 islands in Galápagos with recorded presence of *P*. *downsi*. In addition, samples were collected from five collection sites spanning the two islands. While our study provides initial insight into whether avian parasites or pathogens are present in *P*. *downsi* populations, we did not have a large number of samples from multiple islands and locations, so true representation of natural populations may not be fully achieved.

For trypanosome detection, we targeted the SSU rRNA gene of the parasite, since it is a commonly targeted gene and GenBank provides many deposited sequences from this gene to access for sequence comparison and parasite identification using our sequencing data. However, we acknowledge that we could target other genes of interest, as the literature includes studies targeting the glycosomal glyceraldehyde phosphate dehydrogenase (gGAPDH) gene (Wilfert et al., 2011; Fermino et al., 2013) and the spliced leader (SL) RNA gene (Westenberger et al., 2004) for trypanosomatid testing, although adequate numbers of sequences for comparison may not be available for all of these genes.

In conclusion, our results indicate an absence of avian parasites and pathogens we screened for in *P*. *downsi* samples tested. This study adds to the growing body of literature on the ecology of *P*. *downsi*. Many studies focus on the relationships between *P*. *downsi*, as an ectoparasite, and avian hosts (Dudaniec and Kleindorfer, 2006); however, the literature currently lacks studies on *P*. *downsi* as an avian disease vector. We recommend that further research should be conducted to determine whether *P*. *downsi* could be a competent vector of the avian parasites currently in Galápagos. Future studies on *P*. *downsi* should include

sample collection from additional sites and other islands and *P*. *downsi* larval testing, as well as testing for other avian parasites, pathogens, and arboviruses.

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Table I: Tests for Pathogens in *Philornis downsi* in Galápagos Islands

Pathogen	Primers	Reaction conditions	References
Haemoproteus & *Plasmodium*	*Outer* HAEMNF (5'CATATATTAAGAGAATT ATGGAG-3') HAEMNR2 (5'-AGAGGTGTAGCATAT CTATCTAC-3') *Inner* HAEMF (5'-ATGGTGCTTTCGATATAT GCATG-3') HAEMR2 (5'GCATTATCTGGATGTGA TAATGGT-3')	*Outer* Denaturation: 94 C for 3 min 20 cycles: 94 C for 30 sec, 50 C for 30 sec, and 72 C for 45 sec Final elongation: 72 C for 10 min *Inner* The same PCR conditions are used as above, except 35 cycles were conducted instead of 20 cycles.	Waldenström et al., 2004
Microfilariae	COIintF (5'-TGATTGGTGGTTTTGGTAA-3') COIintR (5'-ATAAGTACGAGTATCAATATC-3')	Thermal conditions are the same as in Merkel et al. (2007), using 45 sec for each annealing step.	Merkel et al., 2007
Trypanosomes	*Outer* S-762(GACTTTTGCTTCCTCTA(A/T)TG) S-763 (CATATGCTTGTTTCAAGGAC) *Inner* S-755 (CTACGAACCCCTTTAACAGCA) S-823 (CGAA(T/C)AACTGC(C/T)CTATCA GC)	*Outer* Thermal cycling conditions follow conditions in Sehgal et al. (2001). *Inner* Thermal cycling conditions also follow conditions in Sehgal et al. (2001); however, for the 35 cycles, 72 C is run for 50 sec instead of 30 sec	Sehgal et al., 2001
Trypanosomes	*Outer* Tryp763 (5'- CATATGCTTGTTTCAAGGAC-3') Tryp1016 (5'-CCCCATAATCTCCAATGGAC3') *Inner* Tryp99 (5'-TCAATCAGACGTAATCTGCC-3') Tryp957 (5'-CTGCTCCTTTGTTATCCCAT-3')	*Outer* Denaturation: 95 C for 5 min 5 cycles: 95 C for 1 min, 45 C for 30 sec, and 65 C for 1 min 35 cycles: 95 C for 1 min, 50 C for 30 sec, and 72 C for 1 min Final elongation: 65 C for 10 min. *Inner* Denaturation: 96 C for 3 min 25 cycles: 96 C for 30 sec, 58 C for 1 min, and 72 C for 30 sec Final elongation: 72 C for 7 min	Valkiūnas et al., 2011
Trypanosomes	R221 (5'-GGTTCCTTCCTGATTTACG-3') Medlin B (5'- TGATCCTTCTGCAGGTTCACCTAC-3')	Denaturation: 94 C for 3 min 30 cycles: 94 C for 30 sec, 55 C for 30 sec, and 72 C for 2 min Final elongation: 72 C for 10 min	Votýpka et al., 2012

Table II: Recipes for trypanosome PCR tests. References indicate primer sources.

PCR component	Concentration	Sehgal et al. (2001)		Valkiūnas et al. (2011)		Votýpka et al. (2012)
		Outer (µl)	*Inner (µl)*	*Outer (µl)*	*Inner (µl)*	*(µl)*
Ex Taq ™ Buffer	10X	2.5	2.5	0.8	1.5	2.5
dNTPs	2.5 mM/ each (Total 10 mM)	2.0	2.0	0.8	1.5	2.0
$MgCl_2$	25 mM	2.0	2.0	0.4	1.0	2.0
Primer 1	10 uM	1.0	1.0	0.6	2.0	1.0
Primer 2	10 uM	1.0	1.0	0.6	2.0	1.0
BSA	-	0.3	0.3	-	-	-
ddH_20	-	15.0	15.0	4.7	14.8	14.3
TaKaRa Ex Taq	-	0.2	0.2	0.1	0.2	0.2
DNA/Amplicon	-	1.0 (DNA)	1.0 (Amplicon)	2.0 (DNA)	2.0 (Amplicon)	2.0 (Amplicon)
Total		25	25	10	25	25

Table III: Trypanosomatid sequences from our *P. downsi* samples and their closest BLAST sequence matches. All sequences were obtained using primers from Votýpka et al. (2012), except for Sample ID denoted with *, obtained using primers from Valkiūnas et al. (2011).

			Closest Match			
Island	Site	Sample ID	Description	Total Score	Identity	GenBank Accession Number
Isabela	Mangrove	P247	*Leptomonas pyrrhocoris* clone G58	2004	99 %	JQ658837.1
Santa Cruz	El Barranco	P041	*Leptomonas pyrrhocoris* clone G58	2282	99 %	JQ658837.1
		P057	*Leptomonas pyrrhocoris* clone G58	2154	99 %	JQ658837.1
		P091	*Leptomonas cf. podlipaevi* isolate 59LI	2136	99 %	EU079124.1
		P322	*Blastocrithidia miridarum* isolate ZM	2084	99 %	EU079128.1
		P361	*Blastocrithidia miridarum* isolate ZM	1917	99 %	EU079128.1
		P287*	*Crithidia confusa* Isolate 320AR	1150	100%	JF717837.1
			Crithidia deanei Strain ATCC 30255	1150	100%	EU079129.1
	Los Gemelos	P116	*Leptomonas pyrrhocoris* clone G58	2102	99 %	JQ658837.1
		P120	*Blastocrithidia miridarum* isolate ZM	1894	98 %	EU079128.1
		P129	*Blastocrithidia miridarum* isolate ZM	2040	99 %	EU079128.1
		P034	*Crithidia bombi*	1834	99 %	FN546181.1
			Leptomonas cf. podlipaevi isolate 59LI	1834	99 %	EU079124.1

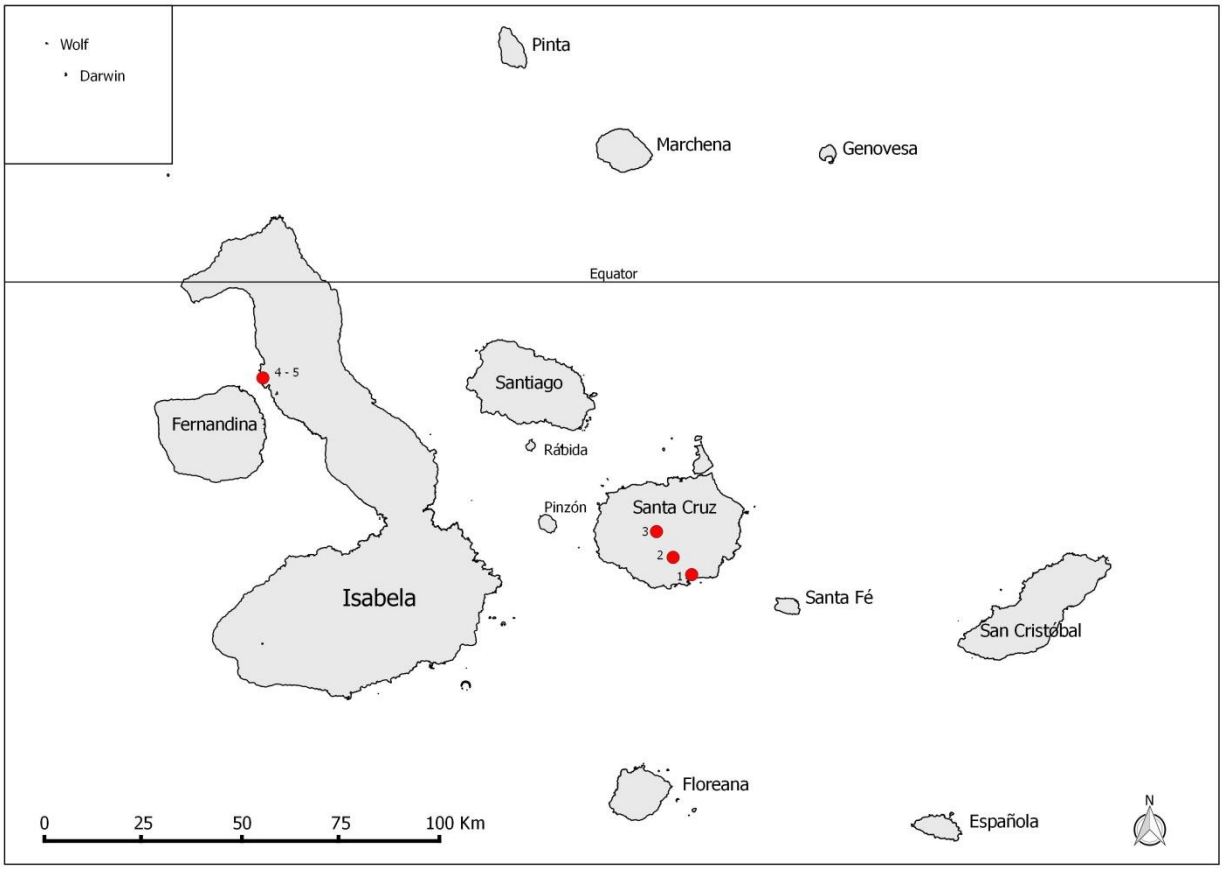

Figure I: Map of Galápagos Islands. Collection sites for *P*. *downsi* samples included (1) El Barranco, (2) Los Guayabillos, (3) Los Gemelos on Santa Cruz and (4) Mangrove and (5) Lava on Isabela.

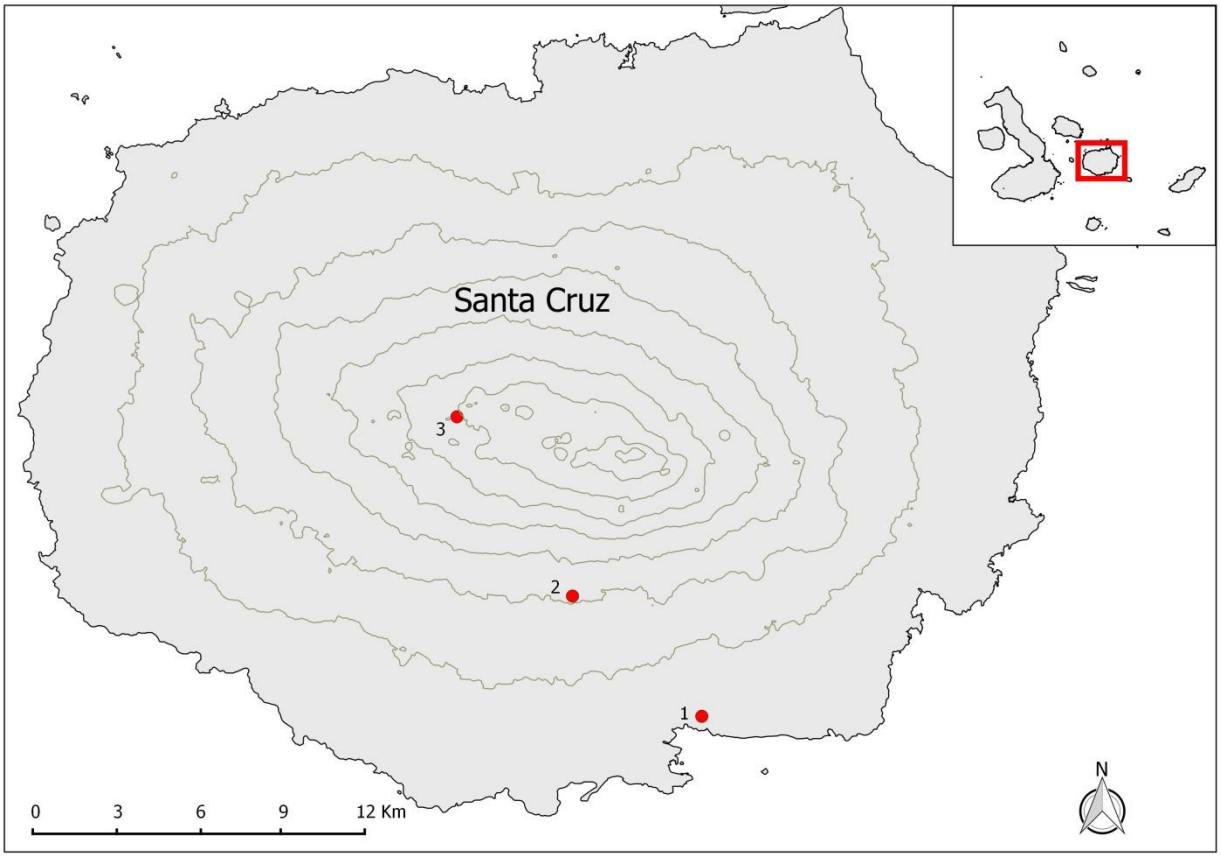

Figure II: Collection sites for *P*. *downsi* samples on Santa Cruz included (1) El Barranco (0˚44’34.1”S, 90˚18’10.4”W), (2) Los Guayabillos (0˚41’68.7”S, 90˚20’78.6”W), and (3) Los Gemelos (0˚ 37’82.0”S, 90˚23’44.4”W). Elevation is indicated with contour lines every 100 meters.

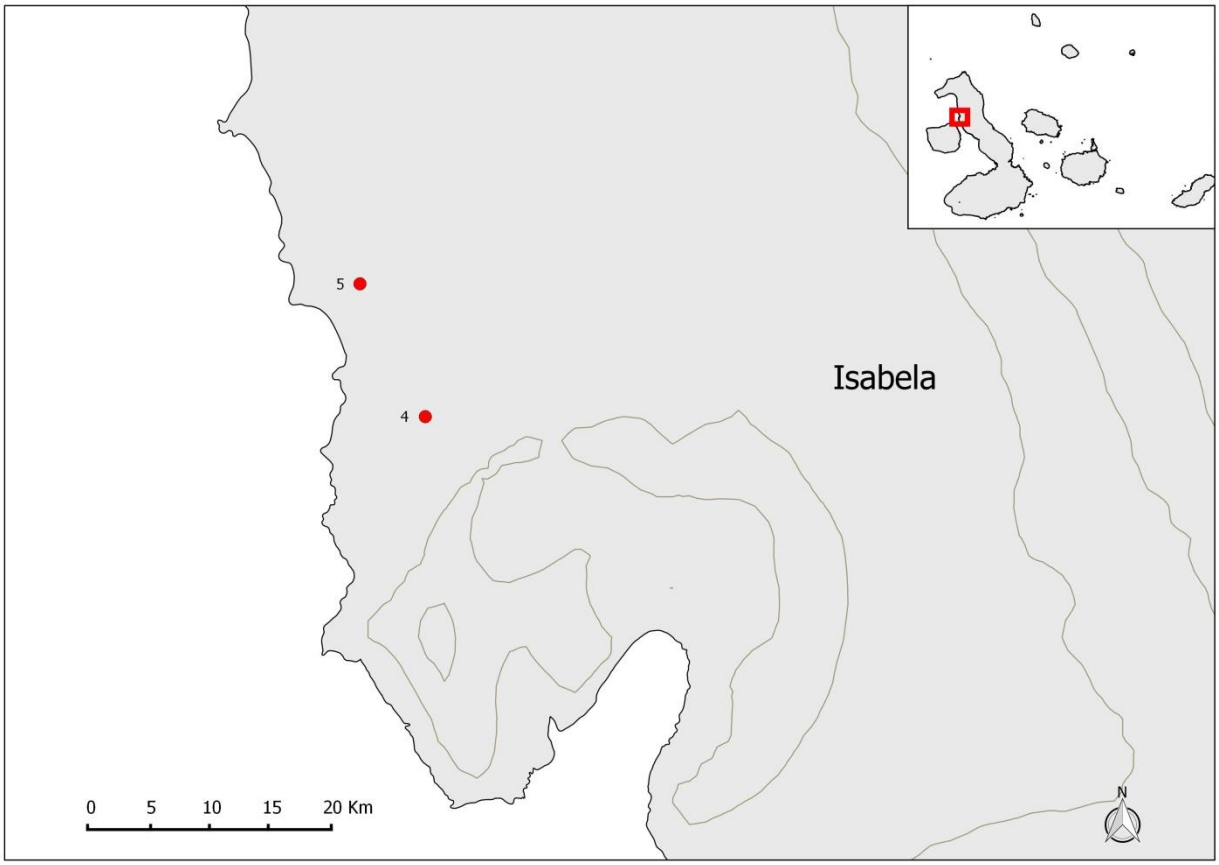

Figure III: Collection sites for *P*. *downsi* samples on Isabela included (4) lava (0°14'46.69"S, 91°23'5.64"W) and (5) mangrove (0°14'32.09"S, 91°23'10.97"W) areas. Elevation is indicated with contour lines every 100 meters.

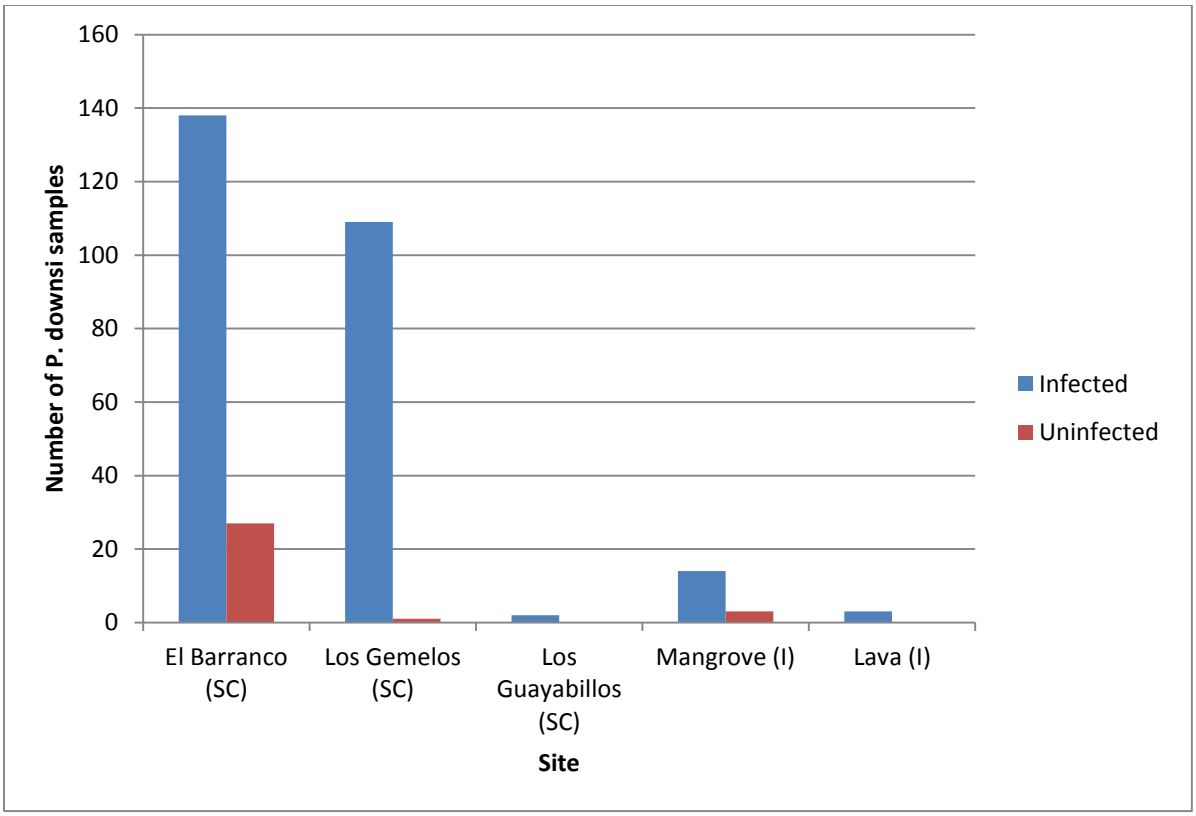

Figure IV: Infection counts for *P*. *downsi* samples tested for trypanosomatids at each collection site. Santa Cruz (SC) and Isabela (I) indicate island of collection. Results displayed were obtained using primers from Sehgal et al. (2001).

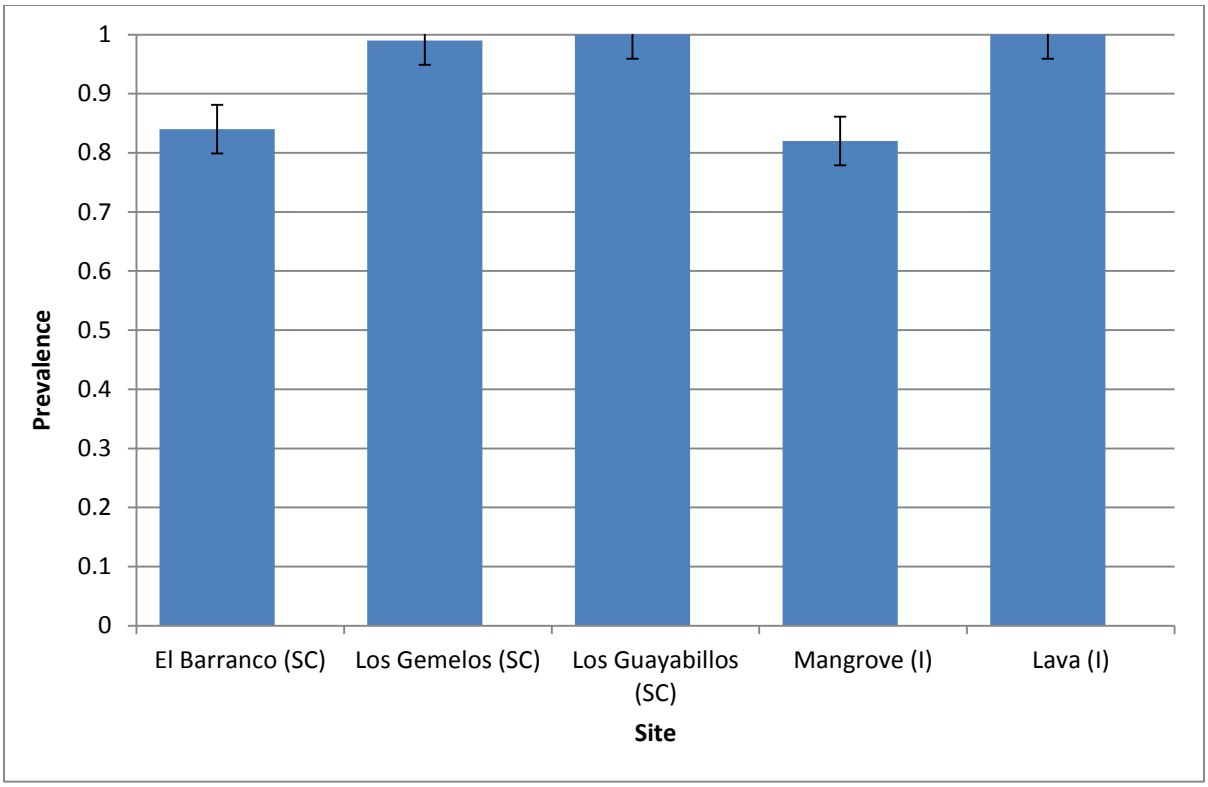

Figure V: Prevalence of trypanosomatid infection in *P. downsi* samples at collection sites within the Galápagos Islands. Santa Cruz (SC) and Isabela (I) indicate island of collection. Results displayed were obtained using primers from Sehgal et al. (2001).

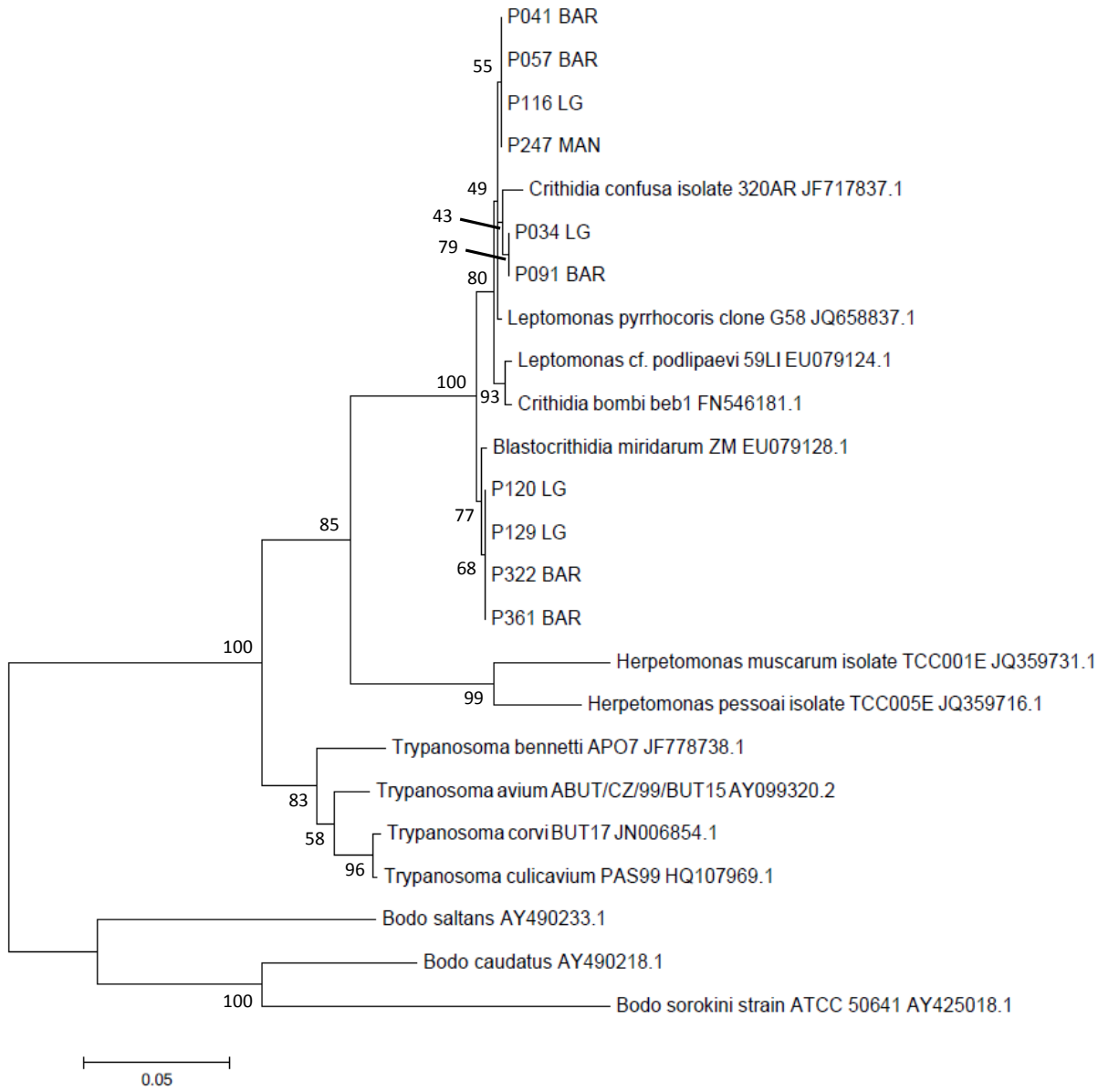

Figure VI: A Maximum Likelihood (ML) phylogenetic estimate of trypanosomatid parasites. Sequences derived from infected *Philornis downsi* samples are indicated by the sample ID (P###), followed by the collection site (BAR = El Barranco, LG = Los Gemelos, MAN = Mangrove). Species names of sequences used for comparison are listed, followed by their GenBank accession numbers. A K2+G model was used to construct the maximum likelihood tree for 940 base pairs of the 18S rRNA gene. Bootstrap values are listed next to nodes. The numbers of site substitutions are indicated by tree branch lengths.