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Galapagos Mosquitoes as Avian Disease Vectors

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ABSTRACT

An avian malaria parasite of the genus *Plasmodium* has been detected consistently in the Galapagos Penguin (*Speniscus mendiculus*) in recent years. Subsequent screening of passerines has revealed more PCR positive birds, with most of them sampled on the southern coasts of Isabela and Santa Cruz islands. We sampled the two zoophilic mosquito species (*Aedes taeniorhynchus* and *Culex quinquefasciatus*) using both CDC light traps and CDC gravid traps along an altitudinal gradient from the southern coasts of these islands to the highlands to investigate whether mosquitoes occur at all elevations, and whether there may be mosquito-free refugia at higher elevation zones as there is in Hawaii. Both species were captured at all sites, except that *C. quinquefasciatus* was not detected at the highest elevation site on Santa Cruz. By PCR screening of pooled mosquito samples, we detected the avian *Plasmodium* parasite lineage infecting the Galapagos Penguin in *Aedes taeniorhynchus* at the lowest elevation site on Isabela, with a prevalence of 0.07% estimated by MIR. In addition, we screened mosquito salivary gland preparations by microscopy, and detected trypanosomes in both mosquito species and on both islands. Further work is needed to determine any threat that these parasites may pose to Galapagos bird populations.

Introduction

Considering arthropod vectors of avian diseases, mosquitoes (Family: Culicidae) are arguably the most important. Female mosquitoes, equipped with

piercing/sucking mouthparts, feed on the blood of vertebrates as a necessary part of their gonotrophic cycle. Through contact with many sequential hosts, female mosquitoes are capable of vectoring an array of both viral and protozoan pathogens of vertebrates. Many of these disease agents are potentially harmful to their host populations, and human activity is capable of magnifying the problem through a number of mechanisms (Daszak et al., 2000). Therefore, monitoring of mosquito populations is essential to understand current threats to avian health, and to establish baseline information for future research.

Avian Malaria in Hawaii

Avian haemosporidians (Sporozoa: Haemosporida) are a phylogenetically distinct group of protozoans, all of which are vectored by dipteran insects (Insecta: Diptera) (Valkiūnas, 2005). Avian haemosporidians of the genus *Plasmodium* are disease agents of mosquito-borne avian malaria. Mosquitoes are cyclopropagative vectors (parasite amplifies and develops in the vector) of avian *Plasmodium* parasites, particularly mosquitoes of the genera *Culex*, *Aedes*, and *Culiseta*, but also *Anopheles* to a lesser extent (Valkiūnas, 2005). While pathogenic effects are limited in avian populations that have coevolutionary associations with the parasite, isolated island populations that are more recently exposed to introduced disease stand at major risk (LaPointe et al., 2012). The most striking example of this is the establishment of avian malaria in the Hawaiian Islands. The introduction of the mosquito *Culex quinquefasciatus* to the archipelago in 1826 set the stage for the transmission of the disease to native birds, causing extinctions and range constrictions of several endemic bird species in the subfamily Drepanidinae (Valkiūnas, 2005; Warner, 1968).

Presence of mosquitoes, once established, is thought to be determined by the heterogeneous distribution of appropriate mosquito habitat across the Hawaiian landscape along an altitudinal gradient (Woodworth et al., 2005). One of the major patterns that has been observed is a decrease in the risk of infection by *Plasmodium relictum* with increasing elevation (Atkinson and LaPointe, 2009; Valkiūnas, 2005; van Riper et al., 1986; Warner, 1968). This has been considered a major determinant of the distributions of many bird species in Hawaii since the introduction of *P. relictum* (Warner, 1968; Valkiūnas, 2005; Scott et al. 1986). Year-round mosquito populations may occur at elevations up to 1500m on the island of Hawaii but seasonal presence of mosquitoes may occur at higher elevations (Goff and van Riper, 1981; LaPointe et al., 2012). Mosquitoes at higher elevations, however, demonstrate a considerably lower level of vector potential due to lower temperatures that inhibit the development of the parasite in the mosquito (LaPointe et al., 2010). Only within the last few decades has a recolonization of the lower elevation forest by the Hawaii amakihi (*Hemignathus virens*) been documented on the island of Hawaii despite the high prevalence of avian malaria parasites and year-round transmission by the *Culex quinquefasciatus* mosquito in this habitat (Woodworth et al., 2005), owed to the evolution of tolerance in the amakihi (Atkinson et al., 2013).

Avian Malaria in Galapagos

An avian blood parasite within the genus *Plasmodium* (lineage A), was recently found in the Galapagos penguin (*Spheniscus mendiculus*), with prevalence ranging from 3 to 9.4% across six field seasons from 2003-2009 (Levin et al., 2009; 2013; Palmer et al., 2013). This is the first known occurrence of any *Plasmodium*

parasite within the archipelago and was detected as part of an ongoing survey effort. Phylogenetic analysis places the parasite in a sister clade with especially pathogenic species including *Plasmodium elongatum* and *Plasmodium relictum*, although the pathogenic effects of lineage A on the penguin are still uncertain. High seroprevalence of malarial antibodies in the population suggests a more widespread exposure among the penguin population than revealed by PCR detection (Palmer et al., 2013). Gametocytes have never been discovered by microscopic examination of blood films, suggesting abortive development of the parasite in the penguin (Levin et al., 2013). If the penguin proves to be a dead-end host for the parasite, there must be an alternative, competent host in which the parasite is completing its life cycle.

The *Plasmodium* lineage infecting the penguin, as well as three additional, distinct *Plasmodium* lineages, have since been found by screening of 3,726 native passerine birds, consisting of 22 passerine species from all major islands (Levin et al., 2013). PCR positive individuals were concentrated among a few sampling locations, suggesting limited transmission zones on Santa Cruz (near Puerto Ayora and Bellavista) and on Isabela (near Puerto Villamil) (Levin et al., 2013). Yellow Warblers (*Dendroica petechial aureola*) on Puerto Villamil, in particular, showed a high prevalence of infection by lineage A (7%), comparable to the prevalence in the Galapagos Penguin at that site (5-7%)(Levin et al., 2013). Lineage A was also detected in one Medium Ground Finch (*Geospiza fortis*) from Santa Cruz Island, giving a prevalence of .2%. Still, PCR-positive passerines lack gametocytes by microscopic examination of blood films, suggesting abortive development (Levin et al., 2013) in these species.

Native Galapagos Mosquitoes

While these parasites have been detected in Galapagos birds, their invertebrate vector(s) remains unknown. There are three species of mosquito in the Galapagos Islands. *Aedes aegypti* was first recorded in the Galapagos in 2001, and occurs only on the islands of Santa Cruz and San Cristobal (Causton et al., 2006). They are highly anthropophilic, and are not suspected to vector avian malaria. *Aedes taeniorhynchus* arrived naturally in the islands approximately 200,000 years ago, and is the only natural arrival of the three mosquito species (Bataille et al., 2009). It is a salt marsh species, and oviposition typically occurs on moist land in areas of temporary inundation near coastlines (Provost, 1951); however, in the Galapagos there is evidence of an isolated population in the highlands far from such typical oviposition sites (Bataille et al., 2009). Recently, the same lineage of *Plasmodium* infecting the Galapagos Penguin was detected by PCR in 25/61 abdomen pools and 1 head/thorax pool of *Aedes taeniorhynchus* from Soccoro Island, Mexico (Carlson et al., 2011). This represents the only known match for this sequence outside of the Galapagos. *Plasmodium* parasites were also detected in a pool of 11 *Culex quinquefasciatus* abdomens from Soccoro Island, although a different lineage than the parasite infecting the Galapagos Penguin (Carlson et al., 2011). These 11 *C. quinquefasciatus* also represent the only specimens that were not identified as *A. taeniorhynchus* (Carlson et al., 2011).

Culex quinquefasciatus was first documented in the Galapagos in 1985 and was most likely introduced with human travel (Whiteman et al., 2005). This species breeds in stagnant fresh water, and its occurrence is thought to be limited to areas of

human establishment (Farajollahi et al. 2011). *Culex quinquefasciatus* is the primary vector of *Plasmodium relictum* in Hawaii (LaPointe et al., 2005), as well as a competent vector for West Nile Virus under experimental conditions (Eastwood et al., 2011) and a suspected mechanical vector for *Avipoxvirus* (Thiel et al., 2005) in the Galapagos archipelago. We predicted that this species is a likely vector of *Plasmodium* in Galapagos as well.

The main objective of this research is to identify possible vectors of *Plasmodium* in the Galapagos Islands. This is the necessary first step toward considering strategies to mitigate disease transmission in the archipelago. Vector incrimination requires the fulfillment of four criteria: (1) A feeding relationship between the suspected vector and the vertebrate host should be demonstrated under field conditions, as well as (2) a spatial and temporal overlap of the arthropod and disease incidence of the vertebrate. (3) The developmental stage infective to the vertebrate host must be repeatedly isolated from field-collected vectors. (4) The suspected vector must be shown to become infected by feeding on the infected vertebrate host and be able to transmit the pathogen between vertebrate hosts under experimental conditions. While a full incrimination is outside of the scope of this project, we aim to identify potential vector populations by fulfilling the second criterion, by isolating the infective disease agents from field-collected arthropods. In addition, we use microscopy as an exploratory tool with which to investigate other disease agents that may be transmissible by Galapagos mosquitoes.

By sampling on two major islands and across the landscape along an altitudinal gradient, we aimed to identify disease-free refugia where mosquitoes do

not occur, and learn about other aspects of the ecology of native mosquitoes that influence the risk of disease transmission in Galapagos.

Methods

Sample Collection

Mosquitoes were collected between May 26 and July 5, 2012, on southern Isabela and southern Santa Cruz. We used the following trap models: New Standard Miniature BlackLight (UV) Trap (Model 1212 John Hock Company, Gainesville, FL), CDC Mini Light Trap with Incandescent Light (Model 2836BQ Bioquip Products, Rancho Dominquez, CA) and CDC Gravid Trap (Model 1712, John Hock Company, Gainesville, FL). Light traps were baited with a CO_2-emitting sugar/yeast/water mixture (250g/35g/2.5L respectively) (Smallegange et al., 2010), which has been shown to increase both catch numbers and diversity, while making the specific trap location less critical (Service, 1977). Gravid traps were baited with a hay-yeast-water infusion according to manufacturer's documentation. These traps were optimized for collecting *Culex* mosquitoes, because standing fresh water is the preferred oviposition medium for this genus. In addition, they target potentially infected individuals, because the traps collect only individuals that have taken blood meals. All traps were set one hour before dusk, and mosquitoes were collected in the early morning (~6:00pm – 6:00am). We established three sites on Isabela, ranging from sea level to ~800m above sea level (ASL) near the top of the Sierra Negra volcano (see Figure 1): Puerto Villamil: 0m ASL (S 00° 57" 17.9', W 90° 58" 20.7'), Zona Agricola: 500m ASL (S 00° 49" 37.9', W 91° 02" 54.5'), and Sierra

Negra: 878m ASL (S 00° 50” 12.5’, W 091° 05” 25.6’). On Santa Cruz, 4 sites were established ranging from sea level to 720m ASL (see figure 2): Puerto Ayora: 0m ASL (S 00° 44” 35.5’, W 090° 18” 09.4’), Bellavista: 183m ASL (W 90° 19” 36.9’), Media Luna: 512m ASL (S 00° 39” 58.9’, W 90° 19” 30.3’), and Cerro Crocker: 720m ASL (S 00° 39” 3.5’, W 90° 19” 35.3’). All sites except for Media Luna were sampled for at least six nights with light traps to guage host-seeking mosquito activity (see Table 1). All mosquitoes were immobilized with chloroform and identified to the species level using morphological characters. Male mosquitoes were identified by morphology, preserved in 95% ethanol, and stored at -20 degrees Celsius at the University of Missouri – St. Louis.

Dissection and Preservation

Immediately after collection, we dissected as many female mosquitoes as time allowed before desiccation prevented dissection. Dissections were performed for preparation of salivary gland smears according to the standard protocol (Valkiunas 2005). The dissection tools were dipped in 10% bleach, rinsed, and wiped dry between dissections. Salivary gland smears were fixed with methanol immediately after drying. All preparations were stained with Giemsa (4mL stock Giemsa/1L phosphate buffer) within 3 weeks of fixation according to Valkiunas (2004). Abdomens and heads/thoraces of these these dissected individuals were pooled separately in 180µL of Longmire’s lysis buffer. Pool sizes varied from 1-9, 5 being most common. Pools contained only individuals that were collected from a single trap. Blood-fed females were preserved individually in 180 µL of lysis buffer and homogenized with a pipet tip for the preservation of blood meals.

Due to time constraints, after dissecting, the remaining individuals were stored in 95% ethanol at -20 degrees Celsius for later processing and DNA extraction in the laboratory at the University of Missouri – St. Louis. For ethanol preserved samples, individuals were placed on a microscope slide for dissection using a stereomicroscope. After removal of legs and wings, a dissecting scalpel was used to sever the abdomen from the head and thorax. The dissection blade was sterilized after each dissection by dipping in 70% ethanol and applying heat with a Bunsen burner. Heads/thoraces and abdomens were pooled in separate microcentrifuge tubes, and ethanol was allowed to evaporate for at least three hours in a fume hood prior to DNA extraction.

Microscopy

We used microscopy in order to detect sporozoites, the life-stage of the parasite that is infective to the vertebrate host. We also consider this as a more comprehensive tool to investigate parasitic infections of the mosquito vectors. Whole salivary gland smears were examined using either an Olympus BH-2 or Olympus CX31 (Olympus, Shinjuku, Tokyo, Japan) with 1000X total magnification to identify the presence of the sporozoites. Time taken to read a salivary gland preparation in it's entirety varied from ~30-60 minutes, depending on the size of the preparation and the amount of artifact present.

DNA Extraction, PCR, and Sequencing

DNA was extracted from female mosquito tissues using Nucleospin Tissue® silica-membrane spin column kits (NucleoSpin Tissue, Macherey-Nagel, Düren, Germany). Tissues were first homogenized with a heat-sealed pipette tip (Njabo et

al., 2009), and extractions were performed according to the manufacturer's protocol, substituting the 180 µL Longmire's lysis buffer for the T1 buffer. For individual abdomen and head/thorax samples, the DNA product was eluted with 45 µL instead of 100 µL to maintain a concentration suitable for PCR screening.

All individuals and pools were screened for *Plasmodium* by nested PCR using the primer sets HAEMF/HAEMR (initial) and HAEMNF/HAEMNR2 (nested) from Waldenström et al. (2004) that target a 580 bp fragment of the parasite's mitochondrial cytochrome b gene (cyt *b*). All reactions were performed according to Waldenström et al. (2004), but with Takara *Ex Taq* DNA polymerase and reagents (Takara, Ōtsu, Japan). A PCR positive DNA sample from a Galapagos penguin infected with *Plasmodium* was used in all reactions as a positive control. A negative control consisting of all PCR reagents but no DNA was used in all reactions. All second-round amplicons were run on a 2% agarose gel stained with GelStar (Lonza, Rockland, ME) at 90V for 90 minutes. Positive samples were identified by the presence of a band of ~525 base pairs in length.

Positive amplicons were purified using Exonuclease I and Antarctic Phosphatase (#M0289S and #M0293S, New England Bio Labs, Ipswich, Massachusetts). DNA was bi-directionally sequenced using the primers HAEMNF and HAEMNR2 (Waldenström et al., 2004) on an ABI 3130 Genetic Analyzer with BigDye Terminator v3.1 Cycle Sequencing Chemistry (Applied Biosystems, Life Technologies, Carlsbad, California) at the University of Missouri – St. Louis. Forward and reverse sequences were edited manually before assembling consensus sequences using LaserGene SeqMan 4.0 software.

For all salivary gland smears on which trypanosomatids were identified, we performed PCR on the corresponding DNA samples (abdomen and head/thorax) targeting 326bp of the small subunit ribosomal RNA gene (SSU rRNA) using the primers and protocols from Sehgal et al. (2001). All reactions were performed using Takara *Ex Taq* DNA polymerase and reagents (Takara, Ōtsu, Japan). All second-round amplicons were visualized on agarose gels as described above. Amplicons were bi-directionally sequenced as described above using the primers S755 and S823 (Sehgal et al., 2001). Sequences were aligned using the ClustalW algorithm (Larkin et al., 2007) with MEGA 5.2.2 software (Tamura et al., 2011). Primer regions were manually removed from sequences.

Results

Mosquito Occurrences

We sampled mosquitoes using both light traps and gravid traps at 3 elevations on Isabela and 4 elevations on Santa Cruz (Table 1). We collected a total of 2,794 *C. quinquefasciatus* and 1,873 *A. taeniorhynchus* at four sites on Santa Cruz and three sites on Isabela during this season (Table 2). Both species occurred at all elevations, with the exception that *C. quinquefasciatus* was not collected at the high elevation site on Santa Cruz (Table 2). No *Aedes aegypti* mosquitoes were collected. We use the number of mosquitoes collected per trap-night as a measure of relative abundance in our sampling sites (Figure 3). Relative abundance varied for both trap types between elevations. By far the most mosquitoes captured per site were *C. quinquefasciatus* using gravid traps in Bellavista, Santa Cruz. These traps averaged

278 124 *C. quinquefasciatus* mosquitoes per trap-night at this site, while other traps types
279 at all other sites averaged between 0 and 33 mosquitoes per trap-night (Figure 3).
280 **PCR Screening for *Plasmodium***
281 1,927 field-dissected mosquitoes (1,248 *C. quinquefasciatus* and 679 *A.*
282 *taeniorhynchus*) were preserved in 613 head/thorax and 613 abdomen pools, with
283 pool sizes ranging from 1-9 individuals. Of these individuals were able to dissect 294
284 *A. taeniorhynchus* and 640 *C. quinquefasciatus* for preparation of salivary glands
285 smears. For 3 *A. taeniorhynchus* individuals, slides were prepared, but DNA samples
286 were not.
287 The remaining collected specimens were not dissected due to time constraints
288 (1,191 *A. taeniorhynchus* and 1,546 *C. quinquefasciatus*), and were preserved in
289 ethanol. These samples were dissected in the laboratory and extracted as 203
290 head/thorax and 203 abdomen pools containing from 1-24 individuals.
291 We screened all 4,891 mosquitoes (1979 *A. taeniorhynchus* and 2912 *C.*
292 *quinquefasciatus*) by PCR, in 1,888 samples of individual or pools of mosquitoes.
293 PCR screening for *Plasmodium* produced one positive result. This positive sample
294 was a pool of 5 *A. taeniorhynchus* abdomens, collected from a light trap in Puerto
295 Villamil on the island of Isabela in 2012. This gives a prevalence of 0.07% for this
296 site and season by MIR estimation (Black et al. 2005). Amplicon from this reaction
297 is a 100% match with the *Plasmodium* lineage (Lineage A) infecting the Galapagos
298 penguin (Levin et al. 2009), and the *Aedes taeniorhynchus* pool from Socorro Island,
299 Mexico (Carlson et al., 2011). The sequence has been deposited in GenBank
300 (accession number XXXX). The corresponding head/thorax pool for these

individuals was screened, but did not amplify. There are no salivary gland smears from the PCR positive *A. taeniorhynchus* abdomen pool. This positive sample has been reamplified consistently, being used in all subsequent reactions as an additional positive control, diluted 1:10.

Microscopy

Salivary gland smears were prepared from 640 *C. quinquefasciatus* and 294 *A. taeniorhynchus* in the field (Table 3). Sporozoites of *Plasmodium* were not identified on any smears. We did identify the presence of epimastigotes of trypanosomatids on 4 slides. One of these slides was from an *A. taeniorhynchus* mosquito collected in Puerto Villamil, Isabela. The remaining 3 were identified in preparations from *Culex quinquefasciatus* individuals; one individual was collected in Puerto Villamil, Isabela, and the remaining two were collected in Puerto Ayora, Santa Cruz.

Discussion

The main purpose of this study is to identify potential associations between mosquito populations and *Plasmodium* parasites by isolating the infective stage of the parasite, fulfilling the second criteria for vector incrimination. The results from PCR detection in the endemic passerine bird species indicated potential transmission zones for lineage A on the southern coasts of Isabela (Puerto Villamil) and Santa Cruz (Puerto Ayora and Bellavista) (Levin et al., 2013). Both of these locations are near human establishment, which appears to be important for the larval development of *C. quinquefasciatus* because of its reliance on fresh water for larval development. These southern coasts are also near inland lagoon areas, which present

the preferred temporary, brackish water medium for oviposition by *A. taeniorhynchus*. Screening of mosquito samples for *Plasmodium* produced one positive result, but due to the lack of microscopic evidence of sporozoites in mosquitoes from that same site, we are unable to interpret this as evidence for the competence of *A. taeniorhynchus* as a vector. We can, however, infer that *A. taeniorhynchus* does blood feed on birds that are infected with the *Plasmodium* Lineage A found infecting the Galapagos penguin, yellow warblers, finches, and, perhaps, a still unidentified reservoir species. This gives us some preliminary fulfillment of the first criteria for vector incrimination, which is the demonstration of a feeding relationship between the suspected arthropod vector and the vertebrate host.

We did obtain an adequate sample size of *C. quinquefasciatus* at this same site. Furthermore, gravid traps collected many *C. quinquefasciatus* collected in Puerto Villamil, so we would expect a higher proportion of infected individuals due to their having taken a blood meal prior to collection. This gives evidence that *C. quinquefasciatus* may not be a vector at this site during the dry season. These data do support previous evidence of Puerto Villamil as a transmission zone for *Plasmodium* lineage A in the Galapagos. In addition to the detection of lineage A in more than one bird species at this site, we now have a detection of this lineage from a field collected mosquito. We conclude and that *A. taeniorhynchus* should be considered a potential vector for this parasite; however, more sampling covering more sites during the wet season may prove more productive for isolating the infective stage of the parasite from a field collected mosquito.

For both *A. taeniorhynchus* and *C. quinquefasciatus*, mosquito capture rates varied considerably among sites (Figure 3). While these trapping data give a rough estimate of mosquito abundance, traps are likely to vary in effectiveness across sites due to differences other than the relative abundances of mosquitoes. Trapping data from Bellavista provides a good example; while light trap data indicates scarcity of *C. quinquefasciatus* at this site, we captured many *C. quinquefasciatus* in our gravid traps. The discrepancies between these data may indicate a lack of attractiveness for our light traps near a density of other potential hosts. This could also indicate that, for *C. quinquefasciatus* at this site, females are more limited by the availability of oviposition sites than available vertebrate hosts. For trapping in urbanized areas, we recommend using a combination of traps that can be used to sample mosquitoes in different stages of the gonotrophic cycle in order to gain a better idea of relative abundances across sites. These data can still provide useful insight into the occurrences of mosquito species at different sites.

Our sampling efforts showed the occurance of both A. taeniorhynchus and C. quinquefasciatus at almost all sites (table 2). Lack of collection of *A. aegypti* is possibly because of the timing of our trapping regime, because *A. aegypti* is a day feeder. Our sampling protocol does not allow us to exclude the possibility that *A. aegypti* was present as well. Currently, more is known of *A. taeniorhynchus* than *C. quinquefasciatus* populations in the Galapagos. Although continental populations of *A. taeniorhynchus* are typically limited to areas within ~6km of the coast (Provost, 1951), in the Galapagos, there appears to be an isolated highland population with very little gene flow with the coastal population as shown by fine-scale population genetic

369 analysis (Bataille et al., 2009). Our sampling efforts, however, show no break in the
370 distribution of *A. taeniorhynchus* mosquitoes from sea level to high elevations along
371 the Sierra Negra volcano during the dry season. Bataille et al. (2010) showed that
372 while coastal population numbers correlated with tide height, the highland
373 populations abundances were correlated with precipitation. Because we sampled
374 during the dry season, these results also indicate that *A. taeniorhynchus* highland
375 populations are active to varying degrees in both wet and dry seasons, leaving no
376 temporal break in the potential for disease transmission at these locations. We
377 conclude that any present or future diseases transmitted by *A. taeniorhynchus* are
378 likely not limited by the distribution of the vector, but by the distributions of their
379 vertebrate hosts on Isabela and Santa Cruz.
380 We were able to sample *C. quinquefasciatus* mosquitoes at all sites
381 (elevations), with the exception of the highest elevation site on Santa Cruz Island
382 (Cerro Crocker, ~720m ASL). This could indicate smaller populations sizes at this
383 elevation. *C. quinquefasciatus* is the primary vector of avian malaria and avian pox
384 in Hawaii, and is likely a vector for avian pox in the Galapagos (Thiel et al., 2005). If
385 there proves an association between populations of *C. quinquefasciatus* and avian
386 malaria and pox in the Galapagos, this may indicate that there is a refuge from these
387 disease threats in the Galapagos at high elevations (>720m ASL) during the dry
388 season. This limitation would leave the avifauna of the Galapagos with very little
389 refuge habitat, especially for species that do not occur in the high elevation habitats.
390 With respect to avian *Plasmodium,* this places a greater importance on the
391 determination of the reservoir host and its distribution within islands.

This is the first study in the Galapagos to investigate the distribution of zoophilic mosquitoes and detect mosquito-transmitted avian pathogens belonging to Plasmodium and *Trypanosoma* genera. The results from this study show that there is probably very little refuge, if any, from the potential disease vectors *A. taeniorhynchus* and *C. quinquefasciatus*. These results also support our previous conclusion of Puerto Villamil, Isabela as a transmission zone for the *Plasmodium* lineage A. While we cannot make conclusions on vector competence at this point, we can conclude that *A. taeniorhynchus* does appear to have a feeding relationship with birds infected with *Plasmodium* in Puerto Villamil, Isabela. We did collect an adequate number of *C. quinquefasciatus* at this same site to detect the parasite in these species, so the lack of detection suggests that this species is not as strongly associated with the parasite at this site during the dry season. Our detection of avian trypanosomes by microscopy indicates that they are present on both of the islands that we sampled, and may be more geographically widespread than *Plasmodium* parasites, although, like *Plasmodium*, their potential for pathogenicity in Galapagos bird populations is still unknown.

Detection of Trypanosomatids in the Galapagos Islands

Addendum to Master's Thesis

Daniel Hartman

Avian Trypanosomes

Avian trypanosomes (genus *Trypanosoma*) represent another vector-borne pathogen present in the Galapagos. These are also parasitic protozoans that exhibit an

obligate heteroxenous life cycle. While there is some evidence of avian trypanosome parasites causing pathogenesis in their hosts (Macfie and Thomson, 1929; Thiroux, 1905, Molyneux et al., 1983), controlled studies are limited and these parasites are widely regarded as benign (Baker, 1976). Avian trypanosomes infect many orders of birds, but most often infect raptors and songbirds (Baker, 1976). Despite their significance to avian health, avian parasites of the genus have been poorly studied, and for the majority of species, information on the complete life cycle is currently unknown (Zidkova et al., 2012). This includes a lack of information on vectors in nature. Black flies (Family: Simuliidae), hippoboscids (Family: Hippoboscidae), mosquitoes (Family: Culicidae), biting midges (Family: Ceratopogonidae), and mites (subclass Acari) have all been suggested as vectors (Baker, 1976; Molyneux, 1977). While experimental transmission of trypanosomes by mosquitoes has been demonstrated (David and Nair, 1955; Bennett, 1961; Chatterjee, 1977; Votýpka et al., 2012) few have been incriminated as vectors in the wild (Zidkova et al., 2012).

In the Galapagos, previous microscopic examination of blood films has resulted in the discovery of a trypanosome trypomastigote in a blood smear prepared from a Galapagos Hawk (*Buteo galapagoensis*) on Santiago Island (Parker et al., 2006). This parasite was detected by ongoing avian health survey work. Although this parasite was not identified to the species level, the screening of several other hawks by PCR produced several results in the possibility of pathogenesis caused by avian trypanosomes is cause for concern. This is the only published detection of avian *Trypanosoma* parasites in Galapagos.

While screening salivary gland smears for malarial sporozoites by microscopy, we detected the presence of trypanosomatid epimastigotes in 4 preparations. Partial sequencing of the SSU rRNA gene for the corresponding head/thorax and abdomen pools yielded 3 unique sequences associated with trypanosomatids. Sequences obtained from the *A. taeniorhynchus* abdomen and head/thorax pools from Puerto Villamil, Isabela, matched those of the monoxeneous *Strigomonas culicis*. We detected epimastigotes in a preparation from a *C. quinquefasciatus* from the same location, and amplified a sequence referred to here as trypA, matching *Trypanosoma culicavium* as well as an isolate from a hippoboscid fly. *TrypA* was also identified from abdomen and head/thorax pools of a smear-positive *C. quinquefasciatus* collected in Puerto Ayora, Santa Cruz. Another smear-positive *C. quinquefasciatus* yielded the trypA sequence in the abdomen pool, but a different sequence in the corresponding head/thorax pool (trypB). The trypA sequence aligns with sequences previously obtained from the blood of 5 Galapagos Hawks (*Buteo galapagoensis*) (unpublished data). The sequence referred to here as trypB produces a 99% match with several other sequences in GenBank, mostly isolates from *Culex tarsalis* mosquitoes.

SSU rDNA sequences match those amplified from blood samples taken from Galapagos Hawks and match closely with avian trypanosome parasites in GenBank, suggesting that avian trypanosomes may be infecting Galapagos birds. Sequences from the three *C. quinquefasciatus* mosquitoes were best aligned with sequences of *Trypanosoma culicavium*; however, it is impossible to identify these *Trypanosoma* to the species level without knowledge of their vertebrate host range (Votýpka et al.,

2012). Because trypA matches with more than one sequences, it is likely that higher resolution gained by longer sequences will give more definitive information on the trypanosomes detected in Galapagos *C. quinquefasciatus* mosquitoes. TrypB produces no 100% matches in GenBank, and is likely a new sequence. This is the first detection of an avian *Trypanosoma* species in a potential arthropod vector in the Galapagos. Understanding the vertebrate host breadth and the transmission cycle should be important, since some *Trypanosoma* species appear to be pathogenic to their vertebrate hosts. In addition to these parasites, *Strigomonas culicis* epimastigotes were detected in a salivary gland smear from an *Aedes taeniorhynchus* mosquito. This species is monoxenous (Maslov et al., 2012), occurring only in the mosquito without an intermediate host, so it should not be considered a threat to vertebrate health in the Galapagos Islands.

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