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Galapagos Mosquitoes as Avian Disease Vectors

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Committee

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25

26 **ABSTRACT**

27 An avian malaria parasite of the genus *Plasmodium* has been detected
28 consistently in the Galapagos Penguin (*Speniscus mendiculus*) in recent years.
29 Subsequent screening of passerines has revealed more PCR positive birds, with most
30 of them sampled on the southern coasts of Isabela and Santa Cruz islands. We
31 sampled the two zoophilic mosquito species (*Aedes taeniorhynchus* and *Culex*
32 *quinquefasciatus*) using both CDC light traps and CDC gravid traps along an
33 altitudinal gradient from the southern coasts of these islands to the highlands to
34 investigate whether mosquitoes occur at all elevations, and whether there may be
35 mosquito-free refugia at higher elevation zones as there is in Hawaii. Both species
36 were captured at all sites, except that *C. quinquefasciatus* was not detected at the
37 highest elevation site on Santa Cruz. By PCR screening of pooled mosquito samples,
38 we detected the avian *Plasmodium* parasite lineage infecting the Galapagos Penguin
39 in *Aedes taeniorhynchus* at the lowest elevation site on Isabela, with a prevalence of
40 0.07% estimated by MIR. In addition, we screened mosquito salivary gland
41 preparations by microscopy, and detected trypanosomes in both mosquito species and
42 on both islands. Further work is needed to determine any threat that these parasites
43 may pose to Galapagos bird populations.

44

45 **Introduction**

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

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

56 **Avian Malaria in Hawaii**

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

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

89 **Avian Malaria in Galapagos**

90 An avian blood parasite within the genus *Plasmodium* (lineage A), was
91 recently found in the Galapagos penguin (*Spheniscus mendiculus*), with prevalence
92 ranging from 3 to 9.4% across six field seasons from 2003-2009 (Levin et al., 2009;
93 2013; Palmer et al., 2013). This is the first known occurrence of any *Plasmodium*

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

104 The *Plasmodium* lineage infecting the penguin, as well as three additional,
105 distinct *Plasmodium* lineages, have since been found by screening of 3,726 native
106 passerine birds, consisting of 22 passerine species from all major islands (Levin et al.,
107 2013). PCR positive individuals were concentrated among a few sampling locations,
108 suggesting limited transmission zones on Santa Cruz (near Puerto Ayora and
109 Bellavista) and on Isabela (near Puerto Villamil) (Levin et al., 2013). Yellow
110 Warblers (*Dendroica petechial aureola*) on Puerto Villamil, in particular, showed a
111 high prevalence of infection by lineage A (7%), comparable to the prevalence in the
112 Galapagos Penguin at that site (5-7%)(Levin et al., 2013). Lineage A was also
113 detected in one Medium Ground Finch (*Geospiza fortis*) from Santa Cruz Island,
114 giving a prevalence of .2%. Still, PCR-positive passerines lack gametocytes by
115 microscopic examination of blood films, suggesting abortive development (Levin et
116 al., 2013) in these species.

117 **Native Galapagos Mosquitoes**

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

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

140 human establishment (Farajollahi et al. 2011). *Culex quinquefasciatus* is the primary
141 vector of *Plasmodium relictum* in Hawaii (LaPointe et al., 2005), as well as a
142 competent vector for West Nile Virus under experimental conditions (Eastwood et al.,
143 2011) and a suspected mechanical vector for *Avipoxvirus* (Thiel et al., 2005) in the
144 Galapagos archipelago. We predicted that this species is a likely vector of
145 *Plasmodium* in Galapagos as well.

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

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

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

165

166 **Methods**

167 **Sample Collection**

168 Mosquitoes were collected between May 26 and July 5, 2012, on southern
169 Isabela and southern Santa Cruz. We used the following trap models: New Standard
170 Miniature BlackLight (UV) Trap (Model 1212 John Hock Company, Gainesville,
171 FL), CDC Mini Light Trap with Incandescent Light (Model 2836BQ Bioquip
172 Products, Rancho Dominguez, CA) and CDC Gravid Trap (Model 1712, John Hock
173 Company, Gainesville, FL). Light traps were baited with a CO₂-emitting
174 sugar/yeast/water mixture (250g/35g/2.5L respectively) (Smallegange et al., 2010),
175 which has been shown to increase both catch numbers and diversity, while making
176 the specific trap location less critical (Service, 1977). Gravid traps were baited with a
177 hay-yeast-water infusion according to manufacturer's documentation. These traps
178 were optimized for collecting *Culex* mosquitoes, because standing fresh water is the
179 preferred oviposition medium for this genus. In addition, they target potentially
180 infected individuals, because the traps collect only individuals that have taken blood
181 meals. All traps were set one hour before dusk, and mosquitoes were collected in the
182 early morning (~6:00pm – 6:00am). We established three sites on Isabela, ranging
183 from sea level to ~800m above sea level (ASL) near the top of the Sierra Negra
184 volcano (see Figure 1): Puerto Villamil: 0m ASL (S 00° 57' 17.9', W 90° 58'
185 20.7'), Zona Agricola: 500m ASL (S 00° 49' 37.9', W 91° 02' 54.5'), and Sierra

186 Negra: 878m ASL (S 00° 50' 12.5', W 091° 05' 25.6'). On Santa Cruz, 4 sites were
187 established ranging from sea level to 720m ASL (see figure 2): Puerto Ayora: 0m
188 ASL (S 00° 44' 35.5', W 090° 18' 09.4'), Bellavista: 183m ASL (W 90° 19' 36.9'),
189 Media Luna: 512m ASL (S 00° 39' 58.9', W 90° 19' 30.3'), and Cerro Crocker:
190 720m ASL (S 00° 39' 3.5', W 90° 19' 35.3'). All sites except for Media Luna were
191 sampled for at least six nights with light traps to gauge host-seeking mosquito activity
192 (see Table 1). All mosquitoes were immobilized with chloroform and identified to the
193 species level using morphological characters. Male mosquitoes were identified by
194 morphology, preserved in 95% ethanol, and stored at -20 degrees Celsius at the
195 University of Missouri – St. Louis.

196 **Dissection and Preservation**

197 Immediately after collection, we dissected as many female mosquitoes as time
198 allowed before desiccation prevented dissection. Dissections were performed for
199 preparation of salivary gland smears according to the standard protocol (Valkiunas
200 2005). The dissection tools were dipped in 10% bleach, rinsed, and wiped dry
201 between dissections. Salivary gland smears were fixed with methanol immediately
202 after drying. All preparations were stained with Giemsa (4mL stock Giemsa/1L
203 phosphate buffer) within 3 weeks of fixation according to Valkiunas (2004).
204 Abdomens and heads/thoraces of these these dissected individuals were pooled
205 separately in 180µL of Longmire's lysis buffer. Pool sizes varied from 1-9, 5 being
206 most common. Pools contained only individuals that were collected from a single
207 trap. Blood-fed females were preserved individually in 180 µL of lysis buffer and
208 homogenized with a pipet tip for the preservation of blood meals.

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

219 **Microscopy**

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

228 **DNA Extraction, PCR, and Sequencing**

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

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

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

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

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

265

266 **Results**

267 **Mosquito Occurrences**

268 We sampled mosquitoes using both light traps and gravid traps at 3 elevations
269 on Isabela and 4 elevations on Santa Cruz (Table 1). We collected a total of 2,794 *C.*
270 *quinquefasciatus* and 1,873 *A. taeniorhynchus* at four sites on Santa Cruz and three
271 sites on Isabela during this season (Table 2). Both species occurred at all elevations,
272 with the exception that *C. quinquefasciatus* was not collected at the high elevation
273 site on Santa Cruz (Table 2). No *Aedes aegypti* mosquitoes were collected. We use
274 the number of mosquitoes collected per trap-night as a measure of relative abundance
275 in our sampling sites (Figure 3). Relative abundance varied for both trap types
276 between elevations. By far the most mosquitoes captured per site were *C.*
277 *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

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

305 **Microscopy**

306 Salivary gland smears were prepared from 640 *C. quinquefasciatus* and 294 *A.*
307 *taeniorhynchus* in the field (Table 3). Sporozoites of *Plasmodium* were not identified
308 on any smears. We did identify the presence of epimastigotes of trypanosomatids on
309 4 slides. One of these slides was from an *A. taeniorhynchus* mosquito collected in
310 Puerto Villamil, Isabela. The remaining 3 were identified in preparations from *Culex*
311 *quinquefasciatus* individuals; one individual was collected in Puerto Villamil, Isabela,
312 and the remaining two were collected in Puerto Ayora, Santa Cruz.

313

314 **Discussion**

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

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

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

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

360 Our sampling efforts showed the occurrence of both *A. taeniorhynchus* and *C.*
361 *quinquefasciatus* at almost all sites (table 2). Lack of collection of *A. aegypti* is
362 possibly because of the timing of our trapping regime, because *A. aegypti* is a day
363 feeder. Our sampling protocol does not allow us to exclude the possibility that *A.*
364 *aegypti* was present as well. Currently, more is known of *A. taeniorhynchus* than *C.*
365 *quinquefasciatus* populations in the Galapagos. Although continental populations of
366 *A. taeniorhynchus* are typically limited to areas within ~6km of the coast (Provost,
367 1951), in the Galapagos, there appears to be an isolated highland population with very
368 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.

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

408 **Detection of Trypanosomatids in the Galapagos Islands**

409 Addendum to Master's Thesis

410 Daniel Hartman

411

412 **Avian Trypanosomes**

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

415 obligate heteroxenous life cycle. While there is some evidence of avian trypanosome
416 parasites causing pathogenesis in their hosts (Macfie and Thomson, 1929; Thiroux,
417 1905, Molyneux et al., 1983), controlled studies are limited and these parasites are
418 widely regarded as benign (Baker, 1976). Avian trypanosomes infect many orders of
419 birds, but most often infect raptors and songbirds (Baker, 1976). Despite their
420 significance to avian health, avian parasites of the genus have been poorly studied,
421 and for the majority of species, information on the complete life cycle is currently
422 unknown (Zidkova et al., 2012). This includes a lack of information on vectors in
423 nature. Black flies (Family: Simuliidae), hippoboscids (Family: Hippoboscidae),
424 mosquitoes (Family: Culicidae), biting midges (Family: Ceratopogonidae), and mites
425 (subclass Acari) have all been suggested as vectors (Baker, 1976; Molyneux, 1977).
426 While experimental transmission of trypanosomes by mosquitoes has been
427 demonstrated (David and Nair, 1955; Bennett, 1961; Chatterjee, 1977; Votýpka et al.,
428 2012) few have been incriminated as vectors in the wild (Zidkova et al., 2012).

429 In the Galapagos, previous microscopic examination of blood films has
430 resulted in the discovery of a trypanosome trypomastigote in a blood smear prepared
431 from a Galapagos Hawk (*Buteo galapagoensis*) on Santiago Island (Parker et al.,
432 2006). This parasite was detected by ongoing avian health survey work. Although
433 this parasite was not identified to the species level, the screening of several other
434 hawks by PCR produced several results in the possibility of pathogenesis caused by
435 avian trypanosomes is cause for concern. This is the only published detection of
436 avian *Trypanosoma* parasites in Galapagos.

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

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

460 2012). Because trypA matches with more than one sequences, it is likely that higher
461 resolution gained by longer sequences will give more definitive information on the
462 trypanosomes detected in Galapagos *C. quinquefasciatus* mosquitoes. TrypB
463 produces no 100% matches in GenBank, and is likely a new sequence. This is the
464 first detection of an avian *Trypanosoma* species in a potential arthropod vector in the
465 Galapagos. Understanding the vertebrate host breadth and the transmission cycle
466 should be important, since some *Trypanosoma* species appear to be pathogenic to
467 their vertebrate hosts. In addition to these parasites, *Strigomonas culicis*
468 epimastigotes were detected in a salivary gland smear from an *Aedes taeniorhynchus*
469 mosquito. This species is monoxenous (Maslov et al., 2012), occurring only in the
470 mosquito without an intermediate host, so it should not be considered a threat to
471 vertebrate health in the Galapagos Islands.

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