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The ecology and feeding behavior of mosquitoes in the Galapagos Islands

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The ecology and feeding behavior of mosquitoes in the Galapagos Islands

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Abstract

Mosquitoes remain important vectors in transmitting wildlife diseases. This dissertation aims to understand the role that mosquitoes play in transmitting wildlife diseases such as avian malaria, a protozoan parasite belonging to the genus *Plasmodium*. Using data from wild-caught mosquitoes captured in multiple years and across multiple islands on the Galapagos Archipelago, we describe distributional patterns of mosquitoes, their range limits and assess whether there exists a disease-free refuge as occurs in Hawaii. We show that altitudinal ranges for disease transmission of avian malaria may not be bounded by a stable disease-free refuge, since mosquitoes are found at all elevations, and the highest peaks are significantly lower in Galapagos than in Hawaii. Secondly, we investigate the influence of ecological factors on the distribution and abundance of mosquitoes on the inhabited island of Isla Santa Cruz. We show that both *Aedes taeniorhynchus* and *Culex quinquefasciatus*, two of the three mosquito species found in Galapagos, decline with elevation. We also show the influence of statistically significant factors of elevation, temperature and humidity on mosquitoes in Galapagos. This chapter discusses the ecological requirements of the avian malarial parasite and how this may influence disease dynamics in the Galapagos; sampling sites at all elevations were within the optimal temperature range for both mosquito and parasite development. Thirdly, using data from wild-caught mosquitoes from Santa Cruz, we discuss the feeding range of both *A. taeniorhynchus* and *C. quinquefasciatus*. This chapter takes a molecular approach in screening mosquito bloodmeals using vertebrate universal primers. Fourthly, we use a combination of field captured mosquitoes, molecular screening and microscopy in identifying *Plasmodium* parasites and understanding their competence in the disease dynamics of avian malaria in Galapagos. Collectively, these results aim to guide conservation efforts towards managing disease-transmitting mosquito vectors in Galapagos.

Dissertation synopsis

Isolated oceanic islands are known for their high endemism and are considered biodiversity hotspots, with nine times more endemic vertebrate species than mainland regions of the same size (Kier et al. 2009). However, they are simultaneously known to have low species richness compared to mainland areas and their low genetic diversity contributes to their high extinction rates (Frankham 1997). Endemic island wildlife evolve without significant exposure to many types of pathogens and their limited ability to adapt genetically to environmental change, such as global climate change, diseases, and introduced predators and competitors, can be detrimental to their conservation status. A classic example of this phenomenon involves the introduction to Hawaii of the Southern House mosquito, *Culex quinquefasciatus* in the 1820s, along with avian malaria and pox from the random introduction of exotic birds. The co-introduction of novel pathogens aided by a competent vector such as *C. quinquefasciatus* subsequently resulted in dramatic extinctions among endemic Hawaiian avifauna (Warner 1968). This example illustrates the role of introduced species such as disease vectors and their ability to influence an emerging disease outbreak in novel ecosystems. It is therefore crucial to understand the ecological and evolutionary processes that influence disease vectors, particularly in oceanic island ecosystems, to better aid the conservation of endemic wildlife populations. Hence, the overall aims of this research dissertation are to understand the ecological factors that influence the distributions and abundances of mosquitoes on Galapagos as well as understanding how mosquito feeding behavior can influence disease transmission among endemic island wildlife.

The Galapagos Archipelago serves as a natural laboratory and perfect system to understand the aims of this dissertation. Straddling the equator, the archipelago consists of 19 islands, 42 islets and 26 emerging rocks and is volcanic in origin and situated almost 1000 km

from the west coast of mainland Ecuador (Swash and Still 2005). The islands are known for their high endemism which inspired Charles Darwin's theory of evolution by natural selection. In fact, given its status as an iconic natural system, its flora and fauna are well studied and human movements and impacts in the archipelago are at least partly controlled and monitored by the collective efforts of the Galapagos National Park and the Charles Darwin Research Station.

Despite these efforts, studies have shown that the archipelago already hosts arthropod vectors such as *C. quinquefasciatus* along with two other mosquitoes, the yellow fever mosquito (*Aedes aegypti*) and the black salt marsh mosquito (*Aedes taeniorhynchus*). Estimated to have naturally arrived ~200,000 years ago (Bataille et al. 2009), *A. taeniorhynchus* is known to oviposit in brackish water and adult females show strong preference for taking blood meals from reptiles and mammals over birds (Bataille et al. 2012). In contrast, *A. aegypti* and *C. quinquefasciatus* require fresh water for oviposition and have been estimated to have established populations in the archipelago in 2001 and 1985 respectively (Whiteman et al. 2005, Causton et al. 2006). *Aedes aegypti* is highly anthropophilic and has been found in human-inhabited zones such as Santa Cruz. *Culex quinquefasciatus* has been implicated as a vector of West Nile virus in parts of USA (Mackay et al. 2010) and is known to vector *Plasmodium relictum* in Hawaii (Warner 1968, van Riper et al. 1986).

The archipelago is also home to harmful pathogens such as several *Plasmodium* lineages of avian malaria parasites, mainly found in the Galapagos penguins and other passerines (Levin et al. 2009, 2013). Even though rates of infection of avian malaria is low among Galapagos birds, the impacts that *Plasmodium* parasites may have on the physiology and health of birds in the archipelago remain unknown. Furthermore, the role that mosquitoes

play in transmitting avian malaria parasites among Galapagos birds also remains unknown. Other parasites include several from the genus *Haemoproteus*, one of the three genera besides *Plasmodium* and *Leucocytozoon* belonging to the order Haemosporidia (Valkiūnas 2005). Multiple lineages of *Haemoproteus* have been detected in several Galapagos bird species (Padilla et al. 2004, 2006; Levin et al. 2012) and although parasites are often non-pathogenic in adapted avian hosts (Bennett et al. 1993), they can cause severe pathology in non-adapted birds (Olias et al. 2011) and can affect fitness in certain species (Valkiūnas 2005, Moller-Jacobs et al. 2014). Vectors known to transmit these parasites include biting midges belonging to Culicoides (*Ceratopogonidae*) and hippoboscid flies (*Hippoboscidae*) (Valkiūnas 2005, Levin et al. 2012). However, the widespread distribution of mosquitoes in Galapagos where most of these *Haemoproteus* parasites were found suggests the need to investigate their role in disease transmission. In addition, other pathogens observed in natural populations of endemic birds include microfilariae, which were detected in flightless cormorants (*Phalacrocorax harrasii*) and Galapagos penguins (*Spheniscus mendiculus*) and sometimes at high prevalence (Merkel et al. 2007). Microfilariae are the first larval stage of tissue-dwelling filarial nematodes that belong to the family *Onchocercidae* (Anderson 2000). The majority of filarioid infections in birds are considered nonpathogenic (Campbell 1995), although they can have negative health impacts on the fitness of host birds (Morand and Poulin 2000) and particularly if individuals are infected with multiple pathogens (Davidar and Morton 2006). Most filarial nematodes are transmitted by biting flies, including mosquitoes such as *C. quinquefasciatus* and *A. taeniorhynchus* (Erickson et al. 2009).

The presence of threatening pathogens and vectors in Galapagos urges the need to understand ecology of mosquitoes and their role in disease transmission. In the first chapter, I utilize a multiple-year collection of mosquitoes sampled across different elevations and

islands such as Santa Cruz, Isabela and Santiago. The main goal of this chapter is to report the distribution of mosquitoes across different altitudinal gradients and we discuss some underlying ecological factors that may influence these distributional patterns. This is an important first step towards identifying distributional hotspots of both mosquitoes and wildlife diseases. Furthermore, we discuss whether there exists a disease-free refugium or refuge where mosquitoes do not exist, particularly in the context of avian malaria on islands such as Galapagos and Hawaii. In the second chapter, we assess whether local mosquito abundances and distributions are influenced by environmental factors. This chapter uses a combination of environmental data accompanying mosquito collections sampled across 18 different sites on the island of Santa Cruz in 2015. We also discuss ecological requirements of both mosquitoes and parasites such as avian malaria and how this may influence the transmission of wildlife diseases in isolated islands such as Galapagos. In the third chapter, I aim to assess host feeding range of mosquitoes and investigate whether they exhibit preference in feeding on certain host species in Galapagos. Determining the host feeding range of mosquitoes can provide insights into the mosquito's potential role in spreading diseases amongst different taxa. In the fourth chapter, we screen for avian Plasmodium parasites in mosquitoes collected in four field seasons in Galapagos. We use a combination of field data, molecular screening and microscopy to understand the arthropod's role in the disease dynamics of avian malaria in the archipelago. Collectively, the results presented here may provide insights into mosquito vectors which are important in transmitting wildlife diseases. A major goal of these results is to provide scientific research that informs decisions on managing wildlife diseases such as avian malaria in Galapagos.

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Chapter 1

The distribution of mosquitoes across an altitudinal gradient in the Galapagos Islands

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Abstract

An avian malaria parasite (genus *Plasmodium*) has been detected consistently in the Galapagos Penguin (*Spheniscus mendiculus*) and less frequently in some passerines. We sampled three resident mosquito species (*Aedes taeniorhynchus*, *Culex quinquefasciatus* and *Aedes aegypti*) using CDC light and gravid traps on three islands in 2012, 2013 and 2014. We sampled along altitudinal gradients to ask whether there are mosquito-free refugia at higher elevations as there are in Hawaii. We captured both *A. taeniorhynchus* and *C. quinquefasciatus* at all sites; however, abundances differed across islands and years and declined significantly with elevation. *A. aegypti* were scarce, and limited to areas of human habitation. These results were corroborated by two negative binomial regression models which found altitude, year, trap type and island as categorized by human habitation to be significant factors influencing the distributions of both *A. taeniorhynchus* and *C. quinquefasciatus*. Annual differences at the highest altitude in Isabela and Santa Cruz indicate the lack of a stable highland refuge if either species is found to be a major vector of a parasite such as avian malaria in Galapagos. Further work is needed to confirm the vector potential of both species to understand the disease dynamics of avian malaria in Galapagos.

Keywords: Culex, Aedes, avian malaria, distribution, vector, altitude

Introduction

Mosquitoes play an important role in the transmission and disease dynamics of pathogens, particularly on isolated islands where wildlife populations have evolved in the absence of diseases (Warner 1968). The most striking example of this is the establishment of avian malaria in the Hawaiian Islands. Avian malaria describes the disease caused by a phylogenetically distinct group of protozoans belonging to the order Haemosporida and the genus *Plasmodium*, all of which are vectored by mosquitoes (Valkiūnas 2005). The introduction of the mosquito *Culex quinquefasciatus* to the Hawaiian archipelago in 1826 set the stage for the transmission of the disease to native birds, causing extinctions and range constrictions of many endemic bird species in the subfamily Drepanidinae (Valkiūnas 2005, Warner 1968).

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, van Riper et al. 1968, Warner 1968). This has been considered a major determinant of the distributions of many bird species in Hawaii since the introduction of *P. relictum* (Scott et al. 1968, Valkiūnas 2005, Warner 1968). Only within the last few decades has the recolonization of the lower elevation forest by the Hawaii amakihi (*Chlorodrepanis virens*) been documented on the island of Hawaii despite the high prevalence of avian malaria parasites and year-round transmission by the *C. quinquefasciatus* mosquito in this habitat (Spiegel et al. 2006, Woodworth et al. 2005). This phenomenon has been attributed to the evolution of tolerance in the Hawaii amakihi (Atkinson et al. 2013).

In Hawaii, *C. quinquefasciatus* exhibits an altitudinal distribution and seasonality that is driven largely by temperature, which in turn influences the risk of avian malaria (LaPointe 2000). The distribution of *C. quinquefasciatus* mosquitoes is determined by the availability of appropriate mosquito habitat across the Hawaiian landscape along an altitudinal gradient

(LaPointe et al. 2005, Woodworth et al. 2005). Year-round mosquito populations may occur at altitudes up to 1500m on the island of Hawaii but mosquitoes may occur seasonally at higher elevations (LaPointe et al. 2012, van Riper et al. 1986). However, mosquitoes at higher altitudes 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), thereby creating a refuge for native bird populations.

As part of an ongoing survey effort (Parker 2016, Parker et al. 2006), 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 percent 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. However, microscopic evaluations of blood smears showed no gametocytes, which are infective to arthropod vectors, suggesting parasitic abortive development in a dead-end host (Levin et al. 2013). Lineage A of the *Plasmodium* parasite infecting the penguin, as well as three additional, distinct *Plasmodium* lineages, have since been detected in a few passerine species on the archipelago (Levin et al. 2013). PCR positive individuals were concentrated among a few sampling locations, suggesting limited transmission zones on Santa Cruz (on the southern slopes near Puerto Ayora and Bellavista) and on Isabela (on the southern coast near Puerto Villamil). Gametocytes were not detected in passerines by microscopy of blood films, indicating poor adaptation of the parasite to these hosts (Levin et al. 2013) in addition to the penguins. Of the four *Plasmodium* lineages described in Galapagos, only lineage A has been shown to be established and transmitted regularly (Levin et al. 2013), thus confirming the need for disease surveys on the archipelago (Wikelski et al. 2004).

While these parasites have been detected in Galapagos birds, identity of their arthropod vector(s) remains unknown. There are three species of mosquitoes 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 in Galapagos. *Aedes taeniorhynchus* arrived in the islands approximately 200,000 years ago, and is the only natural arrival of the three mosquito species (Bataille et al. 2009a). It is a coastal salt marsh species that typically oviposits on moist land in areas of temporary inundation (Provost 1951); however, in Galapagos there is evidence of an isolated population in the highlands far from such typical oviposition sites (Bataille et al. 2010).

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 generally associated with human establishments (Farajollahi et al. 2011). *C. quinquefasciatus* is the primary vector of *Plasmodium relictum* and likely vector of *Avipoxvirus* in Hawaii (LaPointe et al. 2005). Galapagos mosquitoes have proven to be competent vectors for West Nile Virus under experimental conditions (Eastwood et al. 2011), and the species is a suspected mechanical vector for *Avipoxvirus* (Thiel et al. 2005).

Fortunately, Galapagos has not experienced a major extinction of native bird populations as in Hawaii. Thus, there is an urgency to understand the disease dynamics of malarial transmissions. Here we focus on the vector component, particularly mosquito distributions across an altitudinal gradient. Through repeated sampling on three major islands and across multiple years, we aimed to identify the distribution of local mosquito populations across an altitudinal gradient. We also aimed to identify disease-free refugia where mosquitoes do not occur. This is a necessary first step toward understanding the potential role of disease-transmitting mosquitoes in Galapagos and identifying their distributional hotspots.

Materials and methods

Study site

Located 1000 km west of the coast of Ecuador, the Galapagos archipelago consists of 13 major islands, 19 smaller islands and 42 islets that are volcanic in origin and host high endemism of both plant and animal species. Observations and collections of some of these endemic species inspired naturalist Charles Darwin's theory of evolution by natural selection following his visit in 1835 on the Beagle (Darwin 1839). The islands are volcanic, with a maximum altitude of 1,690 m on the island of Isabela. Even though most of the archipelago is covered in arid, semi xerophytic vegetation due to its location in the Pacific dry belt, the vegetation of this ecoregion is diverse and progresses from the rocky coast, to arid lowlands, transitional, *Scalesia*, *Miconia* and Pampa zones (Perry 1984).

These ecoregions are influenced by the north-south migration of the Inter-Tropical Convergence Zone (ITCZ) (Sachs et al. 2009). The latitudinal shift of the ITCZ interacts with trade winds and ocean currents to produce two climatic seasons, a dry season and a wet season. During the dry season when the ITCZ is north (10°N) of Galapagos, the southeast trade winds create dry conditions mainly along the coast of the archipelago. Sea surface temperatures influence precipitation in Galapagos resulting in distinct microclimates that differ between the coast and the highlands (Trueman and d'Ozouville 2010). For instance, during the dry season, cool air from the ocean surface travel up to higher elevations and becomes trapped below warmer air, creating an inversion layer. This condensation effect results in the formation of a heavy mist called *garua* above 250m and drier conditions on leeward northern slopes (Trueman and d'Ozouville 2010). Thus, the highlands experience consistent precipitation in the dry 'cool or *garua*' season, in contrast to the coastal lowlands which remain dry. The dry season spans from

June to December and long-term weather data from Santa Cruz record average monthly rainfall from 10.4 mm to 32.99 mm and average monthly temperatures from 21.5 °C to 23.8 °C (Charles Darwin Research Center 2017). In contrast, the wet ‘hot’ season spans from January to May and occurs when the ITCZ migrates southward (3°N), north east trade winds predominate and the hot Panama Current prevails. Average monthly rainfall for the wet season ranges between 52.6 mm and 81.6 mm while average monthly temperatures range from 25.1 °C to 26.7 °C (Charles Darwin Research Center 2017).

Sample Collection

We collected mosquitoes during three field seasons: from May 26 to July 5, 2012; June 23 to August 1, 2013; and February 6 to June 7, 2014, on southern Isabela and on the islands of Santa Cruz and Santiago in Galapagos. However, in 2012, samples were solely collected on southern Isabela and southern Santa Cruz and excluded Santiago (Figure 1). In all three years of sampling, we established three sites on Isabela, ranging from sea level to ~800m above sea level (ASL) near the top of the Sierra Negra volcano (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 91° 05' 25.6"). On Santa Cruz, three sites were established ranging from sea level to 500m ASL (Figure 1): Puerto Ayora - 0m ASL (S 00° 44' 35.5", W 90° 18' 09.4"); Bellavista - 180m ASL (S 00° 41' 42.3", W 90° 19' 36.9") and Media Luna - 500m ASL (S 00° 39' 58.9", W 90° 19' 30.3"). On Santiago, two sites were established at 0m ASL (S 00°14' 42.50", W 90° 52' 7.75") and 180 meters ASL (S 00° 11' 39.4", W 90° 49' 25.3").

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 Dominguez, CA) and CDC Gravid Trap (Model 1712, John Hock Company, Gainesville, FL). We used both CDC light traps and Miniature Blacklight (UV) traps interchangeably, due to availability of traps in Galapagos and since both traps attract host seeking mosquitoes (Chun-Xiao et al. 2015; Onyango et al. 2013). Light traps were baited with a CO₂-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 (Silver and Service 2008). Gravid traps were baited with a hay-yeast-water infusion to attract *C. quinquefasciatus* (Reiter 1986). In addition, they target potentially infected individuals, because the traps collect gravid females 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 trapped at each site once per field season for three to six consecutive nights with gravid traps and light traps. All mosquitoes were immobilized with chloroform, sexed, and identified to species level using morphological characters.

Statistical analysis

We were specifically interested in the occurrence of mosquitoes and whether mosquito abundances were influenced by factors such as trap type, year of trapping, altitude and island as categorized by human occupation (inhabited or uninhabited). We constructed two regression models, one for *A. taeniorhynchus*, and one for *C. quinquefasciatus*. *Aedes aegypti* data were not analyzed due to a low sample size (n=10) through all years and sites. We evaluated the abundances of mosquitoes at each site as number of mosquitoes captured divided by trapping effort, which is number of functioning traps multiplied by number of nights each trap was set at each location.

Count data such as mosquito abundances generally follow a Poisson distribution; however, over-dispersion can invalidate the Poisson assumption that variance equals mean (Zuur et al. 2009). To accommodate for over-dispersion in our dataset, we used negative binomial regression models and constructed full models with all the effects. These two species-specific models treated number of mosquitoes as the dependent variable, and trap type, island as categorized by human occupation, year of trapping and altitude as independent variables. Effort was used as an offset to incorporate trapping of mosquitoes per trap night. Model selection incorporated the Akaike's information criterion, AIC (Akaike 1973), which penalizes the addition of parameters (Burnham and Anderson 2002) to choose the best model. Given a set of candidate models, the chosen or best model has the smallest AIC as it estimates the closest to the unknown reality that generated the data (Burnham and Anderson 2004). Additionally, these two negative binomial regression models were confirmed using the Pearson goodness-of-fit test that validated the fit of the data to the model. All statistical tests were performed in R Studio version 0.99 (R Development Core Team 2015) and utilized the MASS package (Venables and Ripley 2002).

Results

Mosquito distribution and abundance

We sampled mosquitoes using both light traps and gravid traps at three altitudes on Isabela, three altitudes on Santa Cruz and two altitudes on Santiago for a total effort of 185 trapnights in 2012, 568 trap-nights in 2013 and 456 trap-nights in 2014 (Table 1a, b and c). In 2012, we collected a total of 2,794 *C. quinquefasciatus* and 1,868 *A. taeniorhynchus* at three sites on Santa Cruz and three sites on Isabela (Figure 1). Fewer mosquitoes were trapped in 2013 with

total of 300 *C. quinquefasciatus*, 840 *A. taeniorhynchus* and an additional 3 individuals of *A. aegypti* at three sites on Santa Cruz, three sites on Isabela and two sites on Santiago (Figure 1).

Similarly, and following the same trapping sites of 2012 and 2013, we collected a total of 6,002 *A. taeniorhynchus*, 2,130 *C. quinquefasciatus* and 7 *A. aegypti* in 2014. *Culex quinquefasciatus* and *A. taeniorhynchus* occurred at all altitudes in 2012 (Table 2a), and in general, numbers of mosquitoes captured decreased with altitude except between Zona Agricola (500m) and Sierra Negra (878m) in Isabela where we caught 2 and 38 *A. taeniorhynchus* respectively (Table 2a). In 2013, neither species was trapped at high altitude (≥ 878 meters) on Isabela but were captured at all sites on Santa Cruz, and only *A. taeniorhynchus* occurred at both altitudes on Santiago (Table 2b). In 2013 and in 2014, we captured a total of 25 individuals of *C. quinquefasciatus* on the coast of the uninhabited island of Santiago (Table 2a, b). In 2014, we did not capture *A. taeniorhynchus* at the highest altitude on Isabela and in Santa Cruz (Table 2c, Figure 2c). However, *A. taeniorhynchus* were present at both altitudes sampled on the island of Santiago (Table 2c). In 2014, *C. quinquefasciatus* was captured at all altitudes on Isabela and on Santa Cruz (Table 2c). Generally, *C. quinquefasciatus* was captured at altitudes of 500m in the inhabited islands in all trapping years (Table 2a, b, c). No *A. aegypti* mosquitoes were collected in 2012 but 3 individuals of this species were caught on the coast of Santa Cruz in 2013. In 2014, we captured an additional 3 *A. aegypti* on Santa Cruz and 4 more on the island of Isabela, with all individuals collected at low altitudes (Table 2b).

We used the number of mosquitoes collected per trap-night as a measure of abundance in our sampling sites. Mosquito abundances varied for both trap types between altitudes in all years of trapping. By far, the highest abundance was observed for *C. quinquefasciatus* using gravid traps in Bellavista, Santa Cruz. In 2012, these traps averaged 124 *C. quinquefasciatus* mosquitoes per trap-night at this site, while other trap types at all other sites averaged between 0

and 63 mosquitoes per trap-night (Figure 2a). In 2013 and 2014, *A. taeniorhynchus* was the most common mosquito captured per site using light traps. In 2013, light traps averaged 5 mosquitoes of *A. taeniorhynchus* captured in Zona Agricola, Isabela and 113 mosquitoes of *A. taeniorhynchus* caught per trap night at this same site in 2014 (Figure 2b, c). Other sites averaged between 0 and 3 mosquitoes per trap-night in 2013 and 0 and 17 mosquitoes per trap-night in 2014.

Factors influencing mosquito abundances

The abundances of *C. quinquefasciatus* did not follow a normal distribution (Shapiro-Wilk test, $W = 0.4245$, $p < 0.001$) and were highly dispersed, therefore the associations of mosquito abundances and independent variables were analyzed using a generalized linear model. Altitude, year of trapping, trap method (light traps) and island as categorized by human inhabitation were statistically significant factors in predicting abundances of *C. quinquefasciatus* (Table 3). The negative binomial regression model found mosquito abundances to be significantly negatively associated with altitude at 500m ($z = -4.739$, $p < 0.0001$) and 878m ($z = -5.328$, $p < 0.0001$), indicating sharp declines in *C. quinquefasciatus* abundances at these altitudes on both Isabela and Santa Cruz (Figure 1). In fact, only three percent of individuals were captured at 500m across all trapping years in Isabela and Santa Cruz. Total numbers of *C. quinquefasciatus* were significantly lower at 878m as we captured only 6 out of 5224 individual mosquitoes on Sierra Negra in Isabela across all trapping years (Table 2a, b, c). Abundances of *C. quinquefasciatus* were negatively associated with trapping years 2013 ($z = -2.710$, $p < 0.001$) and 2014 ($z = -3.442$, $p < 0.05$); only 5 percent of mosquitoes were captured in 2013 (Table 2a, b, c). Light traps also had a significant negative effect on abundances of *C. quinquefasciatus* and only captured 818 mosquitoes across trapping years ($z = -6.131$, $p < 0.0001$), thus indicating CDC

gravid traps as being highly effective in capturing this species. Uninhabited islands as a category of human occupation was also significantly negatively associated with abundance for this species ($z = -5.691$, $p < 0.001$); only 25 individual *C. quinquefasciatus* were captured at the coastal altitude site on Santiago, across all years.

To assess the associations of factors with *A. taeniorhynchus* abundances, we utilized a negative binomial regression model given distribution patterns of mosquitoes deviated from normality (Shapiro-Wilk test, $W = 0.3455$ $p < 0.0001$). Similar to results from our model with *C. quinquefasciatus*, we found all factors (altitude, year of trapping, trap type and island as categorized by human inhabitation) to be significantly associated with abundances of *A. taeniorhynchus* (Table 3). Mosquito abundances were significantly negatively associated with two altitudes, 180m ($z = -5.004$, $p < 0.0001$) and 878m ($z = -3.442$, $p < 0.0001$), thus indicating a decline in *A. taeniorhynchus* with increasing altitude. At 180m, we captured only 40 individuals of *A. taeniorhynchus* at Bellavista in Santa Cruz and 14 *A. taeniorhynchus* in the transition zone of the uninhabited island of Santiago across all trapping years (Table 2a, b, c). Populations of *A. taeniorhynchus* became even smaller with increasing altitudes as we only captured 38 mosquitoes at Sierra Negra (878m) in Isabela in all trapping years. In 2013, mosquito numbers were extremely low and only accounted for 10 percent of total captures across all trapping years ($z = -3.916$, $p < 0.0001$). In contrast, the year 2014 marked the highest captures of *A. taeniorhynchus* and accounted for 69 percent of total captures (Table 2c); they were mainly captured using light traps, thus indicating a significant positive association with mosquito abundances ($z = 3.259$, $p < 0.001$). Uninhabited islands as a category of human inhabitation was also significantly associated with *A. taeniorhynchus* abundances ($z = 1.983$, $p < 0.01$) and accounted for 12 percent of total captures across all islands (Table 2a, b, c, Table 3).

Discussion

This is the first study in the Galapagos to investigate the occurrences and abundances of mosquitoes along an altitudinal gradient across different islands that were sampled in multiple years. Our sampling efforts showed the occurrence of both *A. taeniorhynchus* and *C. quinquefasciatus* at almost all sites, although *A. taeniorhynchus* generally existed in larger populations than *C. quinquefasciatus* and abundances of both species decreased with altitude.

Low collections of *A. aegypti* is possibly because of the timing of our nighttime trapping regime, as *A. aegypti* is a day feeder. Our sampling methods did not allow us to exclude the possibility that *A. aegypti* was present in higher abundances than we detected. Hence, the presence of *A. aegypti* on inhabited islands such as Santa Cruz and Isabela in our study warrants future sampling protocols that account for mosquito species that are daytime feeders.

Our study also detected *C. quinquefasciatus* on the uninhabited island of Santiago. This species has been previously recorded in the urban zones of the four inhabited islands in Galapagos (Bataille et al. 2009b, Causton et al. 2006, Peck et al. 1998, Whiteman et al. 2005). Given that it is a freshwater obligate (Patrick and Bradley 2000), it is assumed to be common in or near areas of human habitations where freshwater is found. In Hawaii, the foraging behavior of feral pigs creates water-filled cavities in tree ferns (Goff and van Riper 1980), facilitating establishment of suitable *C. quinquefasciatus* larval habitats. The presence of *C. quinquefasciatus* on Santiago in 2013 and 2014 indicates that populations are established there, utilizing naturally occurring larval habitats such as water-filled cavities found in mangroves or porous lava rocks on the coast.

In addition, there is evidence that *C. quinquefasciatus* has been repeatedly introduced to the islands from mainland Ecuador via airplanes (Bataille et al. 2009b) since it was first identified in 1985 (Whiteman et al. 2005) and its broad range is attributed to their ability to

exploit several modes of human transportation (Kilpatrick et al. 2004). Its presence on Santiago, an uninhabited island that is not linked by air transportation, suggests that sea transportation could be a major source of entry for freshwater obligates such as *C. quinquefasciatus*. We recommend that control measures to monitor the movement of human-assisted transportation of mosquitoes among islands be implemented and enforced in managing mosquito-borne diseases.

Our sampling efforts demonstrated no break in the occurrence of *A. taeniorhynchus* from coastal to high altitudes on both uninhabited and inhabited islands in Galapagos; however, abundances of mosquitoes differed temporally, across elevations, and among islands. Currently, more is known of *A. taeniorhynchus* than *C. quinquefasciatus* populations in Galapagos. Although continental populations of *A. taeniorhynchus* are typically limited to areas within ~6km of the coast (Provost 1951), in Galapagos there appears to be an isolated highland population as shown by fine-scale population genetic analysis (Bataille et al. 2010). Our sampling efforts in 2012 demonstrated no break in the distribution of *A. taeniorhynchus* mosquitoes from sea level to high altitudes along the Sierra Negra volcano on Isabela or at Media Luna on Santa Cruz (Figure 1). However, populations of *A. taeniorhynchus* were not detected at the highest altitude on Isabela in 2013 and 2014 and on Media Luna in Santa Cruz in 2014.

Generally, *A. taeniorhynchus* abundances decreased significantly with increasing altitude, with the exception of Zona Agricola (500m) in Isabela in 2014. We captured over 3000 individuals (~188 mosquitoes per trap night) of *A. taeniorhynchus* at the local organic dump site in Isabela in 2014, which also acted as a stop-over for introduced cattle egrets (*Bubulcus ibis*) during their daily migration to their roosting site on the coast. Thus, this site would be an ideal candidate for capturing and screening mosquitoes for diseases such as avian malaria, particularly if introduced birds such as *B. ibis* are suspected to be reservoirs.

Our analysis also indicated that trapping year was significantly associated with the abundance of *A. taeniorhynchus* ($p < 0.0001$). Similar studies in Galapagos have indicated that the abundance of *A. taeniorhynchus* differs significantly by season, with more mosquitoes trapped during months of high precipitation (Bataille et al. 2010). We captured *A. taeniorhynchus* at higher altitudes in 2012 but not in 2013 and 2014, thus suggesting the persistence of *A. taeniorhynchus* is determined by temporal abiotic factors that could influence the presence of a highland disease-free refuge.

Currently, the only long-term weather data set existing in Galapagos relies on data collected daily in Puerto Ayora (2m ASL) and Bellavista (180m ASL) in Santa Cruz, and made available by the Charles Darwin Foundation. These data revealed 2014 as the wettest year amongst our sampling seasons with the highest daily precipitation of 23mm and a mean relative humidity ranging from 79 to 96 percent (mean = 87 percent). However, the absence of *A. taeniorhynchus* at higher altitudes in Santa Cruz during the wet season indicates that other abiotic factors besides precipitation could be influencing mosquito populations. High altitudes receive less rain during the wet season due to an interaction of the north-easterly trade winds and hot Panama current (Trueman and d'Ozouville 2010). Thus, precipitation is concentrated mainly on coastal windward facing slopes and conditions conducive for mosquito breeding and survival may be absent in highland altitudes. Also, given the persistence of *A. taeniorhynchus* in drier years at higher elevations, the occurrence of a highland disease-free refuge may be close to impossible; however, this will also depend on the availability of suitable breeding habitats for mosquitoes and conditions that favor mosquito abundance and parasitic development. In addition, a true disease-free refuge will also require that conditions favorable to mosquito and parasitic development will need to be consistently absent from year to year and not only in certain years.

Our trapping efforts also detected no break in the occurrence of *C. quinquefasciatus* on the inhabited islands of Santa Cruz and Isabela, in contrast to Santiago which only had *C. quinquefasciatus* populations occurring at coastal altitudes (0m). Populations of *C. quinquefasciatus* were captured at the coastal to high altitude sites on Santa Cruz and Isabela in 2012. This result was consistent with 2013 and 2014 data, which showed the presence of *C. quinquefasciatus* across all altitudes on Santa Cruz and Isabela, with the exception of Sierra Negra on Isabela in 2013 (Figure 2b).

Generally, the presence of both *C. quinquefasciatus* and *A. taeniorhynchus* at all altitudes in Santa Cruz and Isabela suggests that wind and human transportation could be aiding their dispersal. In Hawaii, mosquito dispersal follows prevailing winds which are generally seaward at night (Freed and Cann 2013; LaPointe 2008). However, during strong trade winds, El Nino storms and rare hurricanes, this dispersal can be upslope (Schroeder 1993) and studies have shown that both *A. taeniorhynchus* and *C. quinquefasciatus* disperse several kilometers with *A. taeniorhynchus* dispersing up to 10 km (Provost 1957) and *C. quinquefasciatus* dispersing up to 3km (LaPointe 2008; Medeiros et al. 2017; Reisen et al. 1991). In Galapagos, capturing *A. taeniorhynchus* and *C. quinquefasciatus* at almost all elevations in the dry seasons of 2012 and 2013 indicates that mosquitoes may be dispersed upslope when prevailing south easterly trade winds move them from the southern windward coast of Puerto Ayora and Puerto Villamil to higher elevations. However, presence of both species on almost all elevations in the wet season of 2014 when north easterly trade winds prevail indicates that mosquitoes could be dispersing from northern leeward coasts to the highlands, and that landscape features such as roads that connect our sampling sites in Santa Cruz and Isabela may be acting as corridors for mosquito movement (LaPointe 2008).

Results from our generalized linear model also indicated *C. quinquefasciatus* abundances were influenced by altitude, and decreased significantly with increasing altitude. Particularly at the highest altitude, abundances declined and became non-existent at times, such as in 2013 on Sierra Negra in Isabela. Long term weather data from Santa Cruz revealed 2013 as being the driest year amongst our sampling seasons. In fact, the highest daily precipitation recorded on the southern coast of Puerto Ayora was 1mm (mean = 0.27 mm), while average relative humidity was 86 percent. Similarly, our sampling season in 2012 coincided with the dry season and the highest daily precipitation recorded was 3 mm (mean = 0.26 mm) while mean relative humidity was 86 percent. Even though southern coastal areas receive less rain in dry years, higher altitudes receive more rain due to a condensation effect where two air masses meet (Colinvaux 1984) and the cool sea surface air is pushed up against the warm land surface air (Hamann 1979). It is in the dry season that higher altitudes experience heavy mist or garua (Trueman and d'Ozouville 2010), with fog condensing on vegetation (Jäger et al. 2009), thereby creating microclimatic habitats similar to a tropical rainforest and providing conditions conducive for mosquito breeding and survival. At least in Santa Cruz, the presence of *C. quinquefasciatus* at higher altitudes in 2013 and 2012 indicates the maintenance of mosquito larval habitats in the dry seasons due to an interplay of abiotic factors such as precipitation, temperature and humidity. However, *C. quinquefasciatus* presence at high elevations in Isabela in the dry season of 2013 highlights the complexity of interaction between abiotic factors on different islands. This also warrants the need for long term sampling of meteorological data at different elevations that coincide with long term mosquito sampling across altitudinal gradients on other islands in addition to Santa Cruz.

Hence, if *C. quinquefasciatus* is the primary vector of avian malaria, a highland disease-free refuge will not exist in many years, given its widespread range across an altitudinal gradient. Our results found year of trapping as a significant factor in influencing *C. quinquefasciatus*

abundances and further provides support that their persistence at higher elevations is temporally variable and likely influenced by seasonal effects such as precipitation and temperature. As temperature decreases with increasing altitude, the development time of mosquito larvae increases (Rueda et al. 1990), and suitable breeding habitats either become scarce or patchily distributed (Goff and van Riper 1980, van Riper et al. 1986). Precipitation also has a direct and indirect effect on malarial transmission by influencing the availability of larval habitats and survivorship of adults. In Hawaii, extended droughts associated with the El Niño Southern Oscillation and extreme rainfall events where areas receive more rain than usual (>200 mm/day) can have a negative effect by causing flooding to mosquito larval habitats and causing adult mortality (LaPointe et al. 2012). In Galapagos, it has been demonstrated that for *A. taeniorhynchus*, tide height and precipitation rather than temperature have a significant effect on both coastal and highland populations found in the island of Santa Cruz (Bataille et al. 2010). However, little is known about the kinds of abiotic factors that influence the persistence of *C. quinquefasciatus* and how this may influence the development and transmission of parasites such as avian malaria.

Perhaps the most important effect of temperature is on extrinsic incubation of avian malaria parasites where lower temperatures at higher altitudes lengthen the development time of *Plasmodium* in mosquitoes. Substantial evidence from human and avian *Plasmodium* species suggests that the parasites can only develop into the infectious stage (sporogony) within mosquitoes at a certain temperature range, suggesting a temperature threshold (Lindsay and Martens 1998, Patz and Reisen 2001). The altitudinal range of avian malaria in Hawaii is limited by the cooler temperatures at high altitude, which inhibit sporogony (LaPointe et al. 2010). The minimum temperature for sporogonic development of *P. relictum* in the mosquito vector *C.*

quinquefasciatus is 13°C. Transmission of *Plasmodium* reaches its peak in the altitudinal range of 900-1500 m, as infective mosquitoes thrive at an altitude where the mean ambient summer temperature is 17°C (LaPointe 2000). Altitudes lower than 900 meters have been marked by large extinctions of native bird populations due to high abundances of vector mosquitoes and temperature favorable to transmitting avian malaria. In Galapagos, the ranges for the transmission zone and the altitudinal range for avian malaria may be much narrower given its small altitudinal range compared to Hawaii. In addition, the altitudinal range for avian malaria in Galapagos may not be bounded by a stable disease-free refuge.

In summary, our results indicate that abundances of *A. taeniorhynchus* and *C. quinquefasciatus* are influenced strongly by altitude, with their populations significantly declining with increasing altitude. Our study shows that even though both species are widespread, there is a temporal effect influencing their annual abundances at higher altitudes. These temporal abiotic factors include temperature and precipitation which directly influence the availability of larval habitats, mosquito abundances, and the sporogony threshold of avian malaria parasites. Hence, if conditions favorable for mosquito and parasitic development are present, this could drive a more intensive epizootic event, especially if there are additional susceptible avian populations at higher altitudes. Thus, we recommend that experimental studies be conducted on both *A. taeniorhynchus* and *C. quinquefasciatus* to determine the abiotic factors that influence occurrence, abundance and persistence of avian malaria parasites at different elevations in Galapagos; this is a critical step towards managing wildlife diseases that pose a threat to endemic avian populations in isolated islands.

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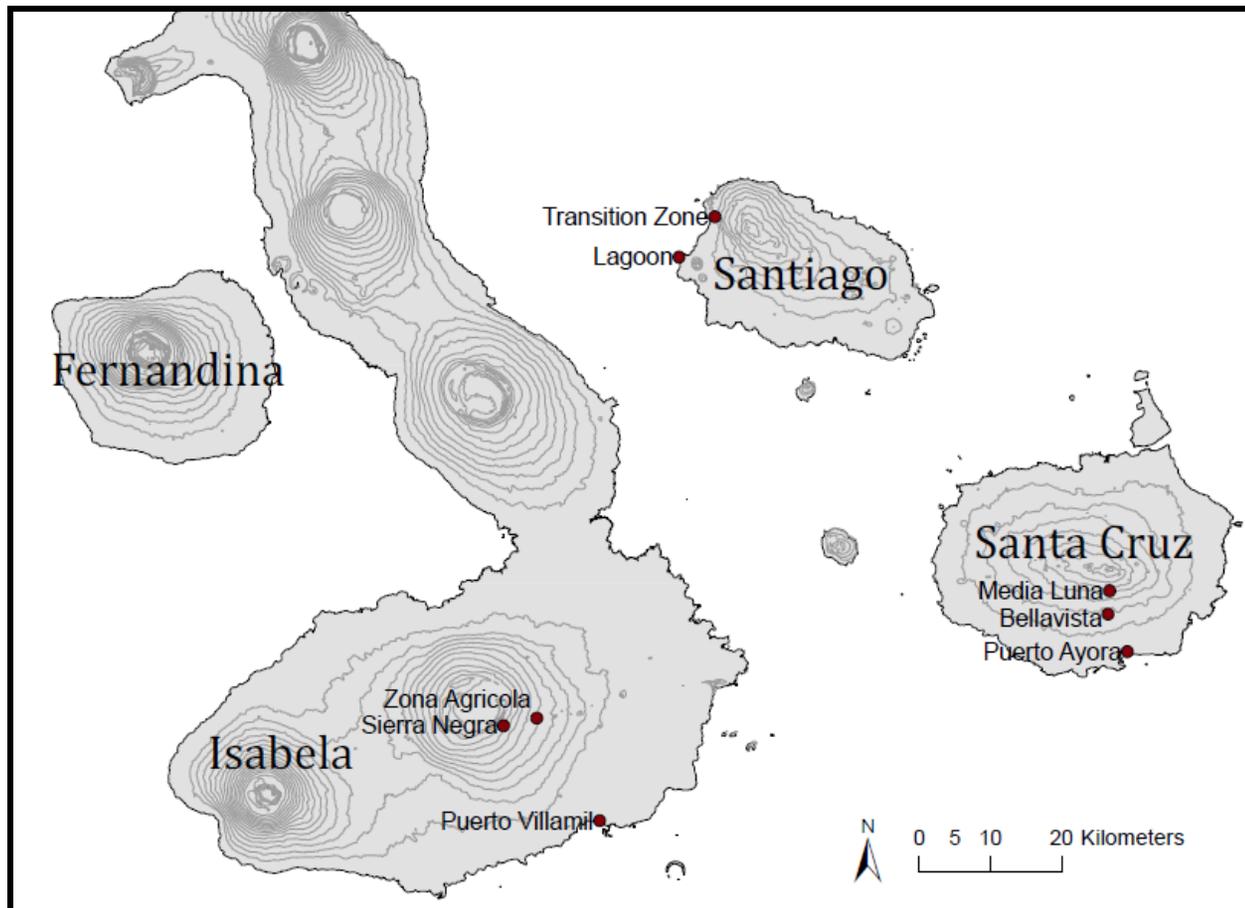
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Tables and Figures

Figure 1. Map of Galapagos with 100m elevation contour lines. Red dots show sampling sites and their elevations for; 1) Santa Cruz - Puerto Ayora (0m), Bellavista (180m) and Media Luna (500m); 2) Isabela - Puerto Villamil (0m), Zona Agricola (500) and Sierra Negra (878) and; 3) Santiago – Lagoon (0m) and Transition zone (180m).



1a. 2012								
	Puerto Villamil, Isabela (0m)	Zona Agricola, Isabela (500m)	Sierra Negra, Isabela (800m)	Totals (Isabela)	Puerto Ayora, S. Cruz (0m)	Bellavista, S. Cruz (180m)	Media Luna, S. Cruz (500m)	Totals Santa Cruz
Light Trap	41	14	14	69	16	12	2	30
Gravid Trap	31	11	13	55	17	12	2	31

1b. 2013											
	Puerto Villamil, Isabela (0m)	Zona Agricola, Isabela (500m)	Sierra Negra, Isabela (878m)	Totals (Isabela)	Puerto Ayora, S. Cruz (0m)	Bellavista S. Cruz (180m)	Media Luna, S. Cruz (500m)	Totals Santa Cruz	Lagoon. Santiago (0m)	Transition zone. Santiago (180m)	Totals Santiago
Light trap	24	48	8	80	64	24	8	96	88	24	112
Gravid trap	24	40	8	72	64	24	8	96	88	24	112

1c. 2014											
	Puerto Villamil, Isabela (0m)	Zona Agricola, Isabela (500m)	Sierra Negra, Isabela (878m)	Totals (Isabela)	Puerto Ayora, S. Cruz (0m)	Bellavista, S. Cruz (180m)	Media Luna, S. Cruz (500m)	Totals Santa Cruz	Lagoon. Santiago (0m)	Transition zone. Santiago (180m)	Totals Santiago
<i>Light trap</i>	36	32	12	80	76	24	12	112	24	12	36
<i>Gravid trap</i>	36	32	12	80	68	32	12	112	24	12	36

Table 1a, b and c. Total trapping effort (Number of days trapped × Number of functioning traps) for 2012, 2013 and 2014 (trap-night are shown for each elevation for each trap typ

2a. 2012									
	Puerto Villamil, Isabela (0m)	Zona Agricola, Isabela (500m)	Sierra Negra, Isabela (878m)	Totals Isabela	Puerto Ayora, S. Cruz (0m)	Bellavista, S. Cruz (180m)	Media Luna, S. Cruz (500m)	Totals Santa Cruz	TOTALS
<i>A. taeniorhynchus</i>	1,354	2	38	1,394	461	1	12	474	1,868
<i>C. quinquefasciatus</i>	1,069	46	4	1,119	153	1,521	1	1,675	2,794
Totals				2, 513				2,154	4,662

2b. 2013												
	Puerto Villamil, Isabela (0m)	Zona Agricola, Isabela (500m)	Sierra Negra, Isabela (878m)	Totals Isabela	Puerto Ayora, S. Cruz (0m)	Bellavista, S. Cruz (180m)	Media Luna, S. Cruz (500m)	Totals (Santa Cruz)	Lagoon. Santiago (0m)	Transition zone. Santiago (180m)	Totals Santiago	TOTALS
<i>A. taeniorhynchus</i>	4	379	0	383	23	19	5	47	409	1	410	840
<i>C. quinquefasciatus</i>	33	91	0	124	149	2	4	155	21	0	21	300
<i>A. aegypti</i>	0	0	0	0	3	0	0	3	0	0	0	3
Totals				507				205			431	1,143

2c. 2014												
	Puerto Villamil, Isabela (0m)	Zona Agricola, Isabela (500m)	Sierra Negra, Isabela (878m)	Totals Isabela	Puerto Ayora, S. Cruz (0m)	Bellavista, S. Cruz (180m)	Media Luna, S. Cruz (500m)	Totals Santa Cruz	Lagoon. Santiago (0m)	Transition zone. Santiago (180m)	Totals Santiago	TOTALS
<i>A. taeniorhynchus</i>	959	3701	0	4,660	676	20	0	696	633	13	646	6,002
<i>C. quinquefasciatus</i>	1,170	21	2	1,193	589	343	1	933	4	0	4	2,130
<i>A. aegypti</i>	4	0	0	4	3	0	0	3	0	0	0	7
Totals				5,857				1632			650	8, 139

Table 2a, b and c. Mosquito samples collected by species, site (elevation) and island for 2012, 2013 and 2014.

Figure 2a, b and c. Number of mosquitoes (N/trap effort) caught per trap night at different elevations in Isabela and Santa Cruz in 2012, 2013 and 2014 using both CDC Light traps (Light) and Gravid traps (Gravid). Black bars represent *A. taeniorhynchus* and grey bars represent *C. quinquefasciatus*.

Figure 2a. Year 2012

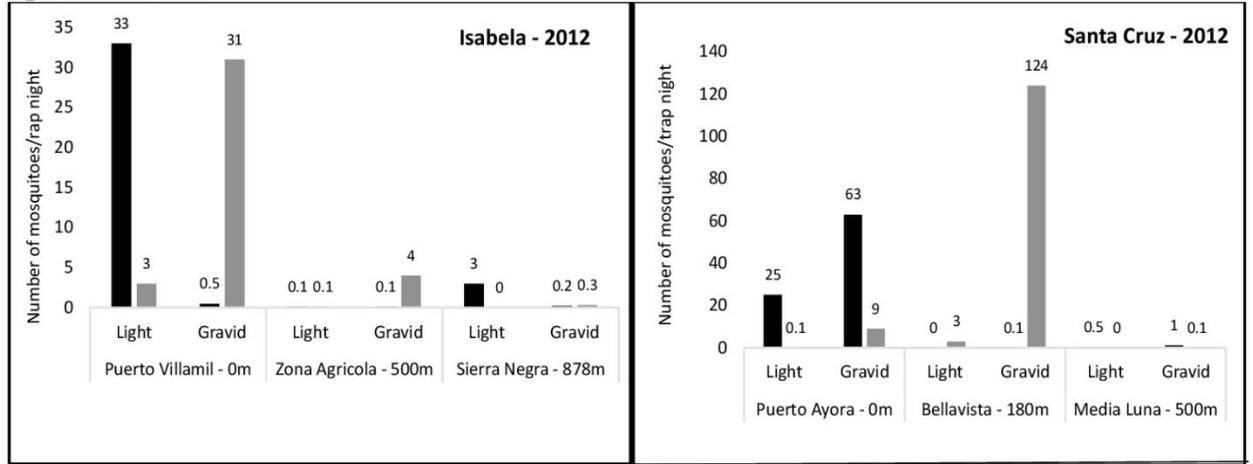


Figure 2b. Year 2013

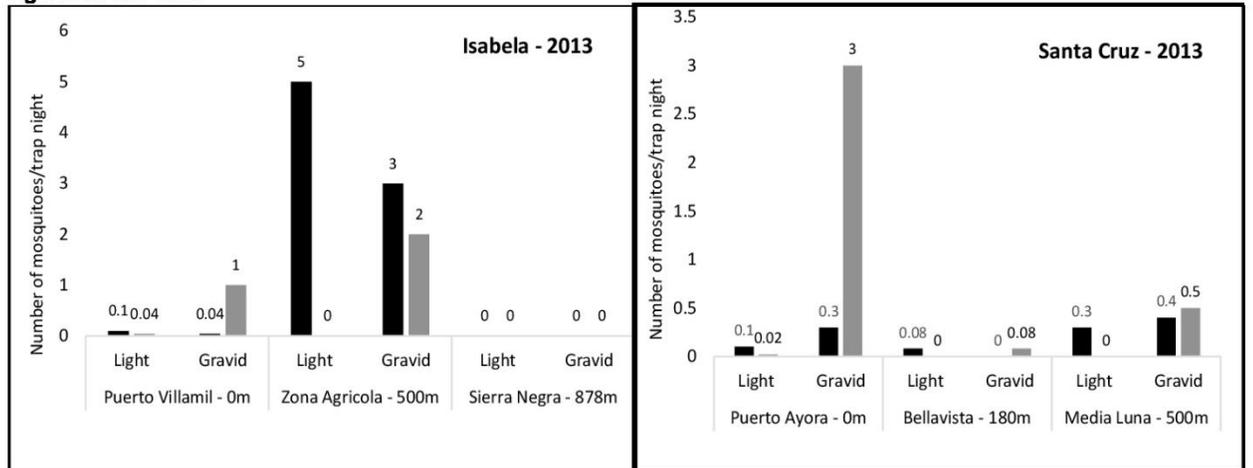


Figure 2c. Year 2014

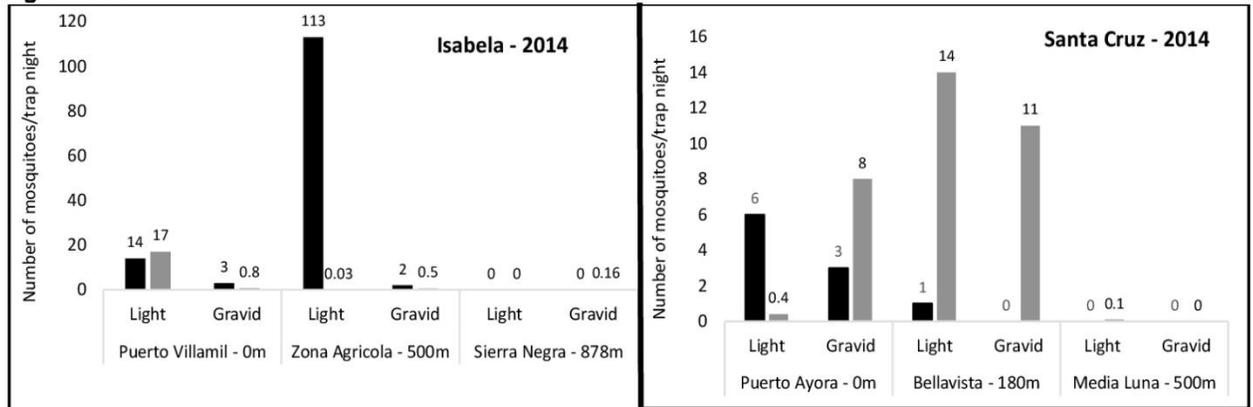


Table 3: Improved Negative Binomial Regression Model with significant factors and AIC.

<i>Culex quinquefasciatus</i>					
	estimate	Std. Err	Z-value	P- value	
Negative binomial regression model: n=4517, AIC = 312 Residual deviance: 40 on 36 df Model fit: $\gamma_2 = 0.296$					
(intercept)	4.8352	0.6587	7.341	2.12e-13	***
Elevation: 500m	-3.1758	0.6702	-4.739	<0.0001	***
Elevation: 878m	-6.5508	1.2296	-5.328	< 0.0001	***
Light trap	-3.4177	0.5574	-6.131	< 0.0001	***
Year 2013	-1.8998	0.7010	-2.710	<0.001	***
Year 2014	-1.2453	0.6739	-1.848	< 0.05	.
Uninhabited island	-5.0891	8.943	-5.691	< 0.0001	***
<i>Aedes taeniorhynchus</i>					
Negative binomial regression model: n=8206, AIC = 381 Residual deviance: 48 on 38 df Model fit: $\gamma_2 = 0.09$					
Intercept	1.0338	0.5963	1.734	< 0.05	.
Elevation 180m	-3.3184	0.6631	-5.004	< 0.0001	***
Elevation 878m	-2.8930	0.8404	-3.442	< 0.0001	***
Year 2013	-2.4763	0.6323	-3.916	< 0.0001	***
Light trap	1.5453	0.4742	3.259	<0.001	**
Uninhabited island	1.3859	0.6989	1.983	<0. 01	*

Best-fit models shown above. Models included mosquito abundances (n) with trap effort as an offset variable. Explanatory terms included year of trapping, category of human habitation (inhabited or uninhabited), elevation and trap type. Only significant variables are included in the model. Asterisks represent significant codes for p values at 0 '***', 0.001 '**', 0.01 '*', 0.05 '.', 0.1 ' '.

Chapter 2

The influence of ecological factors on mosquito abundance and occurrence in Galapagos

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Abstract

This study involves the systematic sampling of mosquitoes across 18 sites established at different elevations and stretching from the north to the south of Isla Santa Cruz, Galapagos. We collected mosquito species *A. taeniorhynchus* and *C. quinquefasciatus*, two commonly occurring species along with environmental variables characteristic of mosquito trapping sites to assess their influence on mosquito abundance and occurrence in the dry season of 2015. We captured *A. taeniorhynchus* at 14 out of 18 sites and captured *C. quinquefasciatus* at low and high elevation sites on Santa Cruz. We utilized two generalized linear models; the first assessed the influence of environmental variables on abundances of *A. taeniorhynchus* and the second assessed the influence of these variables on the presence of *C. quinquefasciatus*. Populations of both mosquito species declined with elevation. Rainfall data were limited, as we sampled during the dry season of 2015. Elevation and maximum humidity were significant in influencing the abundances of *A. taeniorhynchus* while maximum humidity was found to significantly influence the presence of *C. quinquefasciatus*. Both species occurred in sites where temperature, precipitation and humidity should allow for mosquito development as well as parasitic development of the protozoan parasites that cause avian malaria. Further research involving year-round sampling of mosquitoes and accompanying meteorological data as well as experimental studies on vector competence are required to understand disease dynamics of parasites such as avian malaria in Galapagos.

Keywords: Aedes, Culex, Galapagos, Environmental factors, distribution, mosquitoes

Introduction

The genera *Aedes* and *Culex* belonging to the family *Culicidae* are of great medical importance because of their ability to transmit pathogens to humans and wildlife (van Riper et al. 1986, Turell 1999, Farajollahi et al. 2011). Endemic wildlife unique to isolated islands face a higher risk of extinction from parasite introductions given their low genetic diversity compared to mainland relatives and their evolution in the absence of parasites (Frankham 1997, Altizer et al. 2003). A classic example of this phenomenon involves the introduction to Hawaii of the Southern House mosquito, *Culex quinquefasciatus*, in the 1820s, along with the protozoan parasites causing avian malaria and avian pox virus from the random introduction of exotic birds. The co-introduction of novel pathogens aided by a competent vector such as *C. quinquefasciatus* resulted in dramatic extinctions among endemic Hawaiian avifauna (Warner 1968, van Riper III et al. 2002).

In Galapagos, there are currently three mosquito species capable of transmitting human and wildlife pathogens. *Aedes aegypti*, also known as the yellow fever or dengue mosquito, is highly anthropophilic and has been found in human-inhabited islands such as Santa Cruz, San Cristobal and Isabela (Causton et al. 2006, Asigau et al. 2017). Being the most recent of arrivals, it was first detected in 2001 and is known to breed in fresh stagnant water (Causton et al. 2006). In contrast, the native mosquito *Aedes taeniorhynchus* was estimated to have naturally arrived ~200,000 years ago (Bataille et al. 2009b). *Aedes taeniorhynchus* is a widely distributed mosquito in the archipelago, known to oviposit in brackish water such as mangroves and salt marshes on the coast (Provost 1951). However, in Galapagos, its distribution cuts across different elevational gradients from uninhabited highland interiors to human-modified landscapes such as agricultural zones or landfills on the

coast. Its widespread distribution in Galapagos has been associated with environmental factors such as precipitation and its ability to withstand dry conditions (Bataille et al. 2010, Asigau et al. 2017). *Culex quinquefasciatus*, another recent arrival first documented in 1985 (Whiteman et al. 2005), like *A. aegypti*, breeds in fresh stagnant water, is highly anthropophilic and has been associated with the transmission of avian malaria (van Riper et al. 1986). *Culex quinquefasciatus* has been documented on human-inhabited islands such as Santa Cruz and Isabela (Causton et al. 2006). There is recent evidence that its distribution is not limited to low coastal elevations but extends to highland interiors and its abundances are highly influenced by elevation and temporal effects (Asigau et al. 2017).

The islands are home not only to disease-transmitting vectors such as mosquitoes, but to harmful pathogens as well. For instance, extensive screening of avian parasites from six field seasons from 2003 to 2009 resulted in the first discovery of a *Plasmodium* spp. parasite (Lineage A) in the Galapagos penguin (*Spheniscus mendiculus*) (Levin et al. 2009). Parasite prevalence from molecular screening revealed a 3 – 9.4 percent infection across years in penguins found on the coasts of Isabela, Fernandina and Santiago (Levin et al. 2009, 2013). However, microscopic examination of thin blood smears from PCR-positive penguins revealed no gametocytes, the stage of the parasite infective to vector mosquitoes, suggesting parasitic abortive development and that penguins could be dead end hosts (Levin et al. 2013). As part of this ongoing survey (Parker et al. 2006, Parker 2016), our group further sampled 2,923 passerines along major shorelines of Galapagos and three additional *Plasmodium* lineages (Lineage B, C and D) have thus been discovered (Levin et al. 2013). *Plasmodium* parasites were found in yellow warblers, medium ground finch and small ground finch on Fernandina, Santa Cruz and Isabela and evaluations of blood smears from PCR-positive

individuals again revealed no gametocytes, further indicating poor adaptation of the parasite to endemic passerines (Levin et al. 2013). However, the detection of the Plasmodium Lineage A parasite in multiple species sampled on different islands across multiple seasons indicates the establishment of this lineage in the archipelago. Identity of competent vertebrate reservoir hosts and arthropod hosts involved in transmitting avian malaria in Galapagos remain unknown. Thus, research on parasite-vector-host relationship is crucial for managing parasites such as avian malaria and ensuring that major extinctions of endemic birds as occurred in Hawaii are avoided.

Focusing on the arthropod component, we investigated the abundance and distribution of two commonly occurring species, *A. taeniorhynchus* and *C. quinquefasciatus*, with accompanying environmental data across 18 sites on the island of Santa Cruz in Galapagos. Recent research by our group (see Asigau et al. 2017) revealed that abundances of both mosquitoes are influenced by elevation and temporal factors characterized by year of sampling. Here, we follow up to understand the specific ecological factors that influence abundances and occurrence of arthropod vectors that remain potential vectors of parasites such as avian malaria. This is an important step toward identifying disease hotspots and predicting habitats that host endemic wildlife requiring protection and conservation.

Methodology

Study Site

We conducted this study on Isla Santa Cruz, part of the Galapagos archipelago located 1000 km west of the coast of Ecuador. The archipelago hosts numerous endemic flora and fauna species which inspired the formation of Charles Darwin's theory of natural selection

(Darwin 1839, 1859). The Galapagos Islands are volcanic in origin and given their location in the Pacific dry belt, the archipelago is predominantly arid with three distinct ecoregions; the littoral zone, arid zone and humid zone (Perry 1984). The littoral zone is an arid lowland that consists of a narrow stretch of salt tolerant vegetation that fringes the coast of many islands. Salt tolerant vegetation found in this zone include the red mangrove (*Rhizophora mangle*), black mangrove (*Avicennia germinans*), white mangrove (*Laguncularia racemosa*) and button mangrove (*Conocarpus erectus*). The arid zone, found between 80 – 200 meters in elevation (Perry 1984), occupies most of the islands in Galapagos and consists of xerophytic drought tolerant species such as cacti, shrubs, herbaceous plants and trees such as palo santo (*Bursera graveolens*), endemic guayabillo (*Psidium galapageium*) and paga paga (*Pisonia floribunda*). The humid zone follows after the arid or transition zone and is characterized by *Scalesia* (*Scalesia pedunculata*), which are dense shrubs reaching heights of 15m (Mauchamp and Atkinson 2010), *Miconia* (*Miconia robinsoniana*) and the Pampa or fern zone (Kricher 2006).

These diverse ecoregions are influenced by the interaction of trade winds and oceanic currents which respond to the migration of the Inter-Tropical Convergence Zone (ITCZ) (Sachs et al. 2009) to produce two climatic seasons, a wet and dry season. When the ITCZ is 10°N of Galapagos, south east trade-winds strengthen as the south equatorial current (Humboldt Peruvian current) and western Cromwell Current predominate, producing the dry season. This season spans from June to December with average monthly rainfall ranging from 10.4 mm to 32.99 mm (Charles Darwin Research Center 2017). Due to the cool trade winds, the dry season is characterized by cooler monthly temperatures ranging from 21.5 – 23.8 °C (Charles Darwin Research Center 2017) and the interaction between cooler sea surface

temperatures and warmer surface temperatures results in different microclimates experienced at lower and higher elevations, as a result of the leeward-windward effect (Trueman and D'Ozouville 2010). For instance, cool air from the sea travels up to the highlands and causes the warm surface air to sit above it, thereby creating an inversion layer. This condensation effect is characterized by a heavy cloud or mist that envelopes the highlands and occurs above 250 masl with cooler conditions on southern windward slopes and drier conditions on leeward northern slopes (Trueman and D'Ozouville 2010). In contrast, the wet season spanning from January to May is characterized by warmer monthly temperatures ranging from 25.1 °C to 26.7 °C (Charles Darwin Research Center 2017) when the ITCZ migrates 3°S, bringing in the warm Panama Current and northeast trade winds. Average monthly rainfall recorded from long term weather stations on Santa Cruz in the wet season ranges from 52.6 mm to 81.6 mm.

Santa Cruz is the second largest island of the four inhabited islands of Galapagos. Santa Cruz carries the largest human population in the archipelago with over 15 000 inhabitants, which is 62 percent of the total human population of Galapagos (INEC 2010). With an area of 986 km², humans mainly inhabit Puerto Ayora, Miramar, Bellavista, Santa Rosa and Santa Martha, all located on southern windward facing slopes of Santa Cruz. A single 40 km paved road extends from the northern tip at Itabaca Channel right through to the southern tip at Puerto Ayora.

Using this highway as a transect, we established 9 stations (Station 1 – 9), spaced at 5 km equidistant between May 20 and August 3, 2015. Each trapping station comprised two replicates situated on opposite sides of the highway and spaced 300 meters apart, totaling 18 trapping locations across 9 stations (Figure 1). A major advantage of using this highway as a transect is that it passes through the dry northern zone to the wet southern zone of the island

and facilitated mosquito sampling at different elevations. In addition, the headquarters for the Galapagos National Park and Charles Darwin Research Station are based on Santa Cruz, facilitating their involvement during mosquito sampling.

Mosquito trapping

We grouped the 18 trapping locations into northern and southern sites, and randomly selected a northern and southern site for simultaneous trapping sessions lasting three consecutive nights. At each of the 18 trapping locations (Figure 1), we established 4 points measuring 50m apart. We alternated between a CDC light trap (Model 512 John Hock Company, Gainesville, FL) and a CDC Gravid trap (Model 1712 John Hock Company, Gainesville, FL) across the 4 points and set two of each trap type per site. CDC light traps were baited with a concoction of 250g sugar, 35g yeast and 2.5 liters of water to emit CO₂ in luring host-seeking mosquitoes (Smallegange et al. 2010). Gravid traps were baited with hay-yeast-water infusion to attract ovipositing mosquitoes (Reiter 1986). All traps were set one hour before dusk (6:00 pm) and mosquitoes were collected at 6:00 am the next day. Mosquitoes were immobilized with chloroform, sexed and identified to species level using morphological characters.

Environmental variables

A data logger was set at each site daily at 6pm during days of trapping and checked the following morning. Data loggers collected information on environmental variables every 15 minutes and recorded average temperature, maximum temperature, minimum temperature, average humidity, maximum humidity and minimum humidity. A rain gauge was also set at

trapping locations and checked the following morning to record precipitation during mosquito trapping period. We also established a 30m radius from the center of trapping locations and walked transects from the center in each of the four main compass directions (north, east, south and west) and recorded diameter of trees at breast height (DBH) equal to or more than 5cm DBH. All vegetation was identified to species level using morphological characters and height of trees in meters was visually estimated.

Statistical analysis

Since we were interested in the effects of environmental factors on the abundance and occurrence of *A. taeniorhynchus* and *C. quinquefasciatus*, we analyzed the data in two ways. First, count data were fitted to a generalized linear model with a negative binomial distribution and a logit link function to account for non-normality and overdispersion. This analysis was conducted only for *A. taeniorhynchus* because of its larger sample size; *C. quinquefasciatus* were found primarily at lower elevations (6 out of 18 sites) and sample size was inadequate even for zero inflated generalized linear models. Prior to running these regression analyses and models, we explored the data by checking the distribution and variance of response variables using histograms, scatter plots and qqplots. Since trapping effort was standardized in our sampling (i.e., constant number of functioning traps and trapping nights were used at each site), we used total abundance of *A. taeniorhynchus* collected at each site as the dependent - response variable. Our independent variables included average temperature (°C), maximum temperature (°C), minimum temperature (°C), total rainfall per site (mm), average humidity (%), maximum humidity (%), minimum humidity (%), average dbh (cm), average height (m) and elevation (masl). We also checked

for multicollinearity between independent variables by means of Variance Inflation Factor (VIF) and variables with VIFs greater than 3 were removed from the analysis (Zuur et al. 2010). We further applied a backward stepwise regression and eliminated the variable with the highest p value until AIC was minimized, and examined p values for the explanatory variables that remained in the final model. The Akaike's Information criterion - AIC (Akaike 1973) is a statistic which quantifies the goodness-of-fit using a maximum likelihood function and selects a model by penalizing the addition of parameters. Using the principle of simplicity and parsimony (Occam's razor), the best model in comparison to other candidate models has the smallest AIC (Burnham and Anderson 2004) since it best approximates reality given the data. We further validated the fit of the data to the model using the Pearson goodness-of-fit test.

Secondly, since we could not assess the influence of environmental factors on the abundance of *C. quinquefasciatus* due to data limitations, we assessed its occurrence instead using a generalized linear model (GLM). We used binary data (presence or absence) fitted to a binomial distribution with a logit link function. Simple logistic regression models were constructed to assess the effect of each independent variable with occurrence of *C. quinquefasciatus* across sites. Independent variables evaluated included those used in the count data analysis and only those variables with an association of $P < 0.2$ were used in the multiple regression analysis. A backward stepwise approach was applied to the multiple regression analysis and covariates with highest p values were eliminated prior to running subsequent models until AIC was minimized. We examined the p values for the explanatory variables that remained in the final model and validated model fit to the data using the

Pearson goodness-of-fit test. All statistical analyses were performed in R Studio 1.0.153 (R Development Core Team 2015).

Results

General abundance and distribution of mosquitoes

We trapped a total of 757 *A. taeniorhynchus* and 254 *C. quinquefasciatus* across 18 sites in Santa Cruz with a total effort of 216 trap nights (Table 1). Northern sites (Site 4a/b to Site 1a/b; Figure 1) had fewer mosquitoes compared to southern sites (Site 5a/5b to Site 9a/9b) with a total of 207 *A. taeniorhynchus* and 34 *C. quinquefasciatus* captured on northern slopes. *A. taeniorhynchus* were found at all but 4 northern leeward sites with 3A capturing the highest number of mosquitoes (110 mosquitoes) on the northern slope. We also captured a total of 19 individuals of *C. quinquefasciatus* at site 3A. At southern sites, we captured a total of 550 *A. taeniorhynchus* and 220 *C. quinquefasciatus*. Puerto Ayora, the most southern site located on windward slope, yielded 44 percent of total captures of mosquitoes across sites. The highest number of mosquitoes per site was captured at site 9a in Puerto Ayora with a total of 205 *A. taeniorhynchus*. Site 9b located at Charles Darwin Research Station in Puerto Ayora appeared to be a highly favorable site for *C. quinquefasciatus* with 41 percent of total captures and was one of 6 sites in which *Culex* mosquitoes was captured across all 18 sites. Site 9b in Puerto Ayora captured the highest number of *C. quinquefasciatus* with a total of 106 mosquitoes.

Influence of abiotic factors on abundance of *A. taeniorhynchus*

Since abundances of *A. taeniorhynchus* did not follow a normal distribution (Shapiro-Wilk Test, $W = 0.7467$, $p < 0.05$), we assessed associations of environmental variables with mosquito abundances using a negative binomial generalized linear model with a logit link function. VIF analysis resulted in rainfall, maximum temperature, elevation, minimum humidity, maximum humidity and average DBH identified as independent variables. Further model selection utilizing backward stepwise regression was applied to this model and resulted in a final model with elevation and maximum humidity as independent variables and total abundance of *A. taeniorhynchus* per site as the response variable (Table 2).

Elevation was found to be significantly negatively associated with abundances of *A. taeniorhynchus* ($z = -2.645$, $p < 0.001$) (Table 3). Generally, mosquito abundances were highest at ~32 masl (sites 9a and 9b), the two low elevation sites in Puerto Ayora that accounted for 44 percent of captures ($n = 334$ mosquitoes). The most northern sites at Itabaca Channel (sites 1a and 1b) accounted for 96 mosquitoes, the fourth highest site ranked by number of *A. taeniorhynchus* captured. We also captured a total of 114 mosquitoes at Miramar sites 8a and 8b, located 5km from Puerto Ayora and situated at 167 – 170 masl on southern windward facing slopes. Collectively, site 8a and 8b at Miramar favored the second highest captures of *A. taeniorhynchus* (Figure 2). Mosquitoes were also captured at high elevation sites situated at 381 – 618 masl but occurred there in low numbers. We captured a total of 69 mosquitoes at 381-391 masl (site 6a and 6b), two southern sites located 15 km from Puerto Ayora. Mosquito abundances further declined at the highest elevations 618 masl and 595 masl with a total of 20 and 5 mosquitoes at sites 5a and 5b. Interestingly, a total of 110 mosquitoes were captured at site 3a (320 masl) located on the north leeward slope of

Santa Cruz despite no evidence of precipitation during the trapping period (Figure 2, 3). The negative binomial regression model also found maximum humidity to be significantly and positively associated with abundances of *A. taeniorhynchus* ($z = 2.817$, $p < 0.001$; Table 3). Generally, except for southern coastal sites 9a and 9b, mosquito abundances increased with maximum humidity (Figure 4). Puerto Ayora sites captured the highest number of mosquitoes when maximum humidity was as low as 92 percent and reached highs at 98 percent. We captured between 0 – 23 mosquitoes when maximum humidity ranged from 90 – 96 percent. However, when maximum humidity recorded 97 percent to 102 percent, mosquito abundances ranged from 0 to 110 mosquitoes captured per site (Figure 4). Goodness of fit test further validated the fit of the data to the model ($\chi^2 = 0.139$).

Influence of abiotic factors on distribution of *C. quinquefasciatus*

Using simple logistic regression models with a selection criterion of $p < 0.2$, we utilized factors such as maximum humidity ($p = 0.18$), minimum humidity ($p = 0.11$), elevation ($p = 0.17$), rainfall ($p = 0.18$) and average diameter at breast height of trees (DBH) ($p = 0.14$). Model selection using backward stepwise regression revealed elevation, rainfall and maximum humidity as important factors in assessing presence of *C. quinquefasciatus* across sites (AIC = 19.359, Table 4). However, only maximum humidity was found to be significantly associated with presence/absence of *C. quinquefasciatus* (Table 5). Our final model showed that, similar to *A. taeniorhynchus*, *C. quinquefasciatus* were likely to occur at areas of high humidities that ranged from 92 – 101 percent ($z = 2.02$, $p < 0.05$). These sites also averaged a maximum temperature of 30 °C while minimum temperatures dropped as low as 24°C (Figure 5). Even though elevation did not appear as a significant factor for *C.*

quinquefasciatus ($z = -1.718$, $p = 0.08$), sites with both high humidity and an average maximum temperature of 30 °C occurred at low elevation and included 9a and 9b at Puerto Ayora, 1a and 1b at Itabaca Channel, site 3a located on northern leeward facing slopes, and site 5a at Los Gemelos (Figure 5).

Discussion

This study involved the systematic sampling of mosquitoes with accompanying environmental data from 18 sites across all elevations and ecosystems on Isla Santa Cruz, Galapagos. Our sampling in the dry season of 2015 further supports studies that have shown the widespread distribution of *A. taeniorhynchus* and *C. quinquefasciatus* on Santa Cruz (Causton et al. 2006, Bataille et al. 2012, Asigau et al. 2017). Generally, abundances of both species decreased with elevation and at least for *C. quinquefasciatus*, mosquito abundances were mainly concentrated but not limited to coastal low elevations. For both species, abundances of mosquitoes were highest at southern windward facing sites Puerto Ayora and Miramar and on the northern tip of Santa Cruz, Itabaca Channel, which are all located at coastal elevations ranging from 13 – 167 masl. Both Puerto Ayora and Itabaca Channel contain mangrove vegetation which are habitats that *A. taeniorhynchus* favors. However, the widespread distribution of *A. taeniorhynchus* at nearly all elevations indicates that its habitats are not restricted to salt marshes or mangroves found on the coast of Santa Cruz. In fact, larvae of *A. taeniorhynchus* are euryhaline and can tolerate different levels of salinity ranging from 0 to more than 35 percent seawater (Bradley 1994) and pupal mass and larval growth rate are positively influenced by salinity (Clark et al. 2004). In Puerto Ayora resides the largest human population on Santa Cruz and Itabaca Channel is regularly populated with

humans traveling in and out of Santa Cruz, given it is the gateway crossing point to the island from the nearby island of Baltra which contains the airstrip. It is therefore no surprise that *C. quinquefasciatus* highly favors such habitats associated with human populations given its preference for fresh stagnant rain water collected in old tires, ditches, drains, tanks or containers, which are essential for larval development (Teng et al. 1999).

Abiotic factors, particularly precipitation, influence mosquito distribution and abundance (Ahumada et al. 2004, Reisen et al. 2008). Mosquito abundance is an important component of vector capacity and the basic reproductive rate (R_0) (Moller-Jacobs et al. 2014). Therefore, high mosquito abundances may be an indicator for disease hotspots. Since mosquitoes require water bodies to oviposit eggs and for larvae to develop, their abundances and distributions should covary with precipitation. For instance, abundances of *C. tarsalis* in certain regions of California are positively correlated with total precipitation (Reisen et al. 2008). Precipitation has also been found to increase abundances of mosquitoes in arid environments by providing standing water habitats that were not previously available (Vasconcellos et al. 2010). Even in semi-drought conditions, such as wetlands that dry out during drought periods, mosquito abundances flourish following increased precipitation. Dry conditions eliminate mosquito predators and competitors that generally take longer to colonize shared mosquito habitats, thereby allowing habitat generalist and opportunist species such as mosquitoes to quickly re-colonize wetlands following drought periods (Chase and Knight 2003). However, increased precipitation from extreme rainfall events can also result in mosquito mortality by flooding standing water and thereby reducing ideal aquatic habitats required for larval development (LaPointe et al. 2012). Both *A. taeniorhynchus* and *C. quinquefasciatus* larvae feed on microorganisms and detritus and increased precipitation may

dilute or flood these water bodies, making them less favorable for mosquitoes. Results from our analysis did not find any association between precipitation and mosquito abundances. Even though this result may seem inconsistent with the biology and development of mosquitoes, it is not surprising given the mixed results of effects of precipitation on mosquito abundance found in other studies (DeGaetano 2005, De Little et al. 2009, Roiz et al. 2010, Dieng et al. 2012, Bashar and Tuno 2014). Furthermore, precipitation data were restricted to the three-night trapping period at each site in the dry season of 2015 in our sampling scheme. Thus, we were unable to include a lag time in our GLM models since the biology of mosquito larvae development to adulthood exceeds four days. Larvae of both mosquito species usually take 6 to 8 days to develop under optimal conditions (Subra 1981, Tauber et al. 1986). Our sampling period was not only below the optimal time needed for larvae development but since we trapped once at each site, including a lag effect into our regression analysis was unjustified. Thus, we recommend long term mosquito sampling coupled with longer-term meteorological data which are essential for incorporating lag effects into regression models and identifying important abiotic factors and interactions that influence mosquito abundances.

Results from our generalized linear model found *A. taeniorhynchus* abundances were influenced by elevation, and decreased significantly with increasing altitude. Particularly at low coastal elevations, mosquito abundances were highest and this finding could be attributed to available appropriate habitats for larval development such as mangroves for *A. taeniorhynchus*. In addition, *A. taeniorhynchus* occurring on the coast in Galapagos have been known to be positively associated with tide height rather than precipitation (Bataille et al. 2010). Tide height leads to higher population growth rates in this species (Ailes 1998), mainly because depressions in salt marshes are readily filled once tides have receded. Tide

heights also create moist substrates for ovipositing mosquitoes. Mosquitoes can lay more eggs on moist substrates created by tide heights and quickly mature even in dry conditions when rainfall is limited. *A. taeniorhynchus* normally selects substrates which have 70 percent moisture content above the usual saturation of moisture that soils can hold. Eggs laid on substrates with less than 17 percent moisture are highly susceptible to desiccation (Knight and Baker 1962). However, tide height may also bring in predators such as small fish specialized in consuming invertebrates such as mosquitoes (Ritchie and Montague 1995, Ailes 1998). *Aedes taeniorhynchus* has been experimentally shown to avoid ovipositing sites with high concentrations of fish (Ritchie and Laidlaw-Bell 1994) but favor mangrove habitats rich with greater detritus surface and higher organic soil content for larval food (Ritchie and Johnson 1991). Other closely related species such as *A. vigilax*, which breeds in habitats similar to *A. taeniorhynchus* such as saline brackish wetlands, have been observed to reach high abundances in low rainfalls, particularly in the late dry season or early wet season when tide heights are favorable (Yang et al. 2008). Our sampling did not include tide height as a covariate; however, the high abundances of *A. taeniorhynchus* captured at Puerto Ayora, a coastal windward site that experienced low rainfall could indicate interactions of abiotic factors such as rainfall, tide height and elevation.

The presence of mosquitoes at sites 6a and 6b at 380 – 390 masl is not surprising given these sites are located in Santa Rosa, an agricultural and human-inhabited town on Santa Cruz. Furthermore, the presence of *A. taeniorhynchus* even at higher elevations such as sites 5a and 5b (~ 595 – 618 masl) in Los Gemelos reveals the importance of abiotic factors at different elevations. In general, we did not find any effect of precipitation on mosquito abundances, since our sampling was conducted in the dry season. However, capturing

mosquitoes at high elevation sites that experienced heavy rainfall, such as site 5b at Los Gemelos, highlights an important climatic interaction driven by ocean currents and trade winds in Galapagos. Both Santa Rosa and Los Gemelos are located on southern windward slopes and receive precipitation in the form of a heavy mist or garua in the dry season (Trueman and D'Ozouville 2010). This is particularly evident at Los Gemelos, where both cool air from the sea and warm surface air meet to create an inversion layer or garua. This can result in high altitudes receiving increased precipitation in the dry season (Colinvaux and Perry 1984, Trueman and D'Ozouville 2010), thereby creating appropriate microhabitats for mosquito larvae growth and development.

Our model also found maximum humidity to be significantly associated with *A. taeniorhynchus* abundances. High relative humidity can maintain basic survival rate of mosquitoes and induce high hatching rates (Nielsen and Nielsen 1953, Pedrosa et al. 2010). In our study, maximum humidity had a positive effect on *A. taeniorhynchus* abundances when humidity was above 92 percent, although this positive effect could also be due to other complex interactions between climatic factors. High humidity can increase mosquito survival (Clements 2011) but could also be an indication of incoming rainfall which could affect larval growth, larval development, mosquito dispersal and ovipositing positively or negatively, depending on the intensity of precipitation. At the other extreme, eggs laid at low humidity are highly likely to desiccate and adult mosquito longevity is decreased (Wigglesworth 1972, Day 2016). Thus, experimental research on *A. taeniorhynchus* response to extreme weather conditions and how this may influence disease transmission in Galapagos is needed.

Even though we did not find any association between temperature and *A. taeniorhynchus* abundances, many studies have shown its importance in larval development,

adult dispersal and lifespan, and disease transmission (Moore and Bickley 1966, Nayar 1967, 1972, Day 2016). Under controlled environments, *A. taeniorhynchus* eggs readily hatch when exposed to summer temperatures of 23 °C (75 °F) for a week, although mature embryos can undergo facultative diapause when winter temperatures drop as low as -10 °C (14 °F) (Moore and Bickley 1966). Temperature also determines the period spent at pupal stage and is inversely proportional to temperature. For example, the duration of pupal stage at 20.8 °C is 61 hours and 37 hours at 29 °C (Nielsen and Haeger 1954) and adult lifespan of females significantly declines when temperatures reach a high of 32 °C (Nayar 1972). A particularly important effect of temperature is its influence on the incubation of parasites such as avian malaria. Temperatures for sporogonic development of *Plasmodium* species occurs at 16 – 30 °C and ideally at 28 – 30 °C. This means that lower elevations are likely to have temperatures that would encourage the growth of *Plasmodium* parasites in competent arthropod vectors, although temperatures higher than 30 °C may be lethal and temperatures below 16 °C may inhibit parasite development (LaPointe 2000). In Galapagos, low elevation sites (0 – 300 masl), which favored high mosquito abundances in our study, recorded average temperatures ranging from 23 – 25 °C, which is within the range of ideal mosquito and parasitic development of avian malaria. Higher elevation sites such as Santa Rosa and Los Gemelos also averaged temperatures within an ideal range and averaged between 20 – 21 °C. Since *A. taeniorhynchus* occurs at almost all elevations with varying temperatures, it is not surprising that we did not find temperature as a significant factor in influencing *A. taeniorhynchus* abundances. However, its widespread distribution is concerning if it becomes implicated in the transmission of avian malaria, since its temperature requirements for development

coincides with the development of Plasmodium parasites. Experimental studies that test mosquito competence to transmit *Plasmodium* along temperature gradients in Galapagos are needed.

We also found presence of *C. quinquefasciatus* to be significantly influenced by high maximum humidity. Sites which favored high abundances of mosquitoes mainly occurred at lower elevations (0 – 300 masl) where humidities exceeded 92 percent and recorded temperatures ranged from 28 to 30 °C and at 25 °C for site 3A located on the northern leeward slopes of Santa Cruz. Even though temperature was not a significant factor in influencing the occurrence of *C. quinquefasciatus*, high humidities accompanied by high temperatures provide clues of the temperature requirements for this species. *C.*

quinquefasciatus, like *A. taeniorhynchus*, has been found to occur at all elevations in both wet and dry seasons with abundances declining with increasing altitude in Galapagos (Asigau et al. 2017). This arthropod species has been known to successfully transmit avian malaria in the Hawaiian archipelago (van Riper et al. 1986). Development of the avian malaria parasite *P. relictum* is greatly prolonged at 17 °C and ceases at 13 °C (LaPointe 2000), which also coincides with temperatures at 1800 masl in Hawaii, the elevation providing refuge to high densities of endangered and native birds. Above 1800 masl, mosquito breeding habitats become patchy and sparse due to cooler temperatures being non-conducive for larval growth and development, thereby hindering parasitic development and disease transmission in general. In Galapagos, the altitudinal ranges are small compared to Hawaii, with high elevation sites such Los Gemelos (~600 masl) experiencing temperatures within the range of mosquito and parasitic development. Like all mosquito species, larval growth and development is temperature dependent in *C. quinquefasciatus*. Temperature allowing adult

survival and larval growth to adulthood is between the range of 20 – 30 °C and larval development to adulthood decreases significantly as temperatures exceed 27 °C (Rueda et al. 1990). Cooler temperatures below 20 °C and temperatures in excess of 40°C decrease embryonic and larval development times and decrease size of adults (Wall and Shearer 2008). Low coastal elevations in Galapagos usually are characterized by these ambient temperatures and, coupled with sufficient precipitation, can result in high rates of disease transmission, as in Hawaii. In fact, temperature was the main force in driving disease dynamics in Hawaii; at low elevations, fluctuations in mosquito populations were less evident since mosquitoes were able to develop at a broader range of rainfall parameters and reach adulthood at shorter times than at mid or higher elevations (Ahumada et al. 2004). In Galapagos, the presence of *C. quinquefasciatus* at low coastal elevations in Puerto Ayora, Itabaca Channel and even at high elevations such as Los Gemelos indicates that it is widespread. These sites are also characterized by temperatures favorable for mosquito and parasitic development. This is concerning since this species is known to be a competent vector of avian malaria (LaPointe 2000, LaPointe et al. 2012) and if competent avian reservoirs and additional susceptible host populations are found at these elevations, this could result in an epizootic event and threaten the conservation of endemic avifauna in Galapagos.

In conclusion, our results support previous studies that show the widespread distribution of *A. taeniorhynchus* and *C. quinquefasciatus* and the influence of abiotic factors such as elevation, precipitation, humidity and temperature on mosquito abundance and occurrence (Causton et al. 2006, Bataille et al. 2010, Asigau et al. 2017). We found that mosquito abundances generally decline with increasing altitude, both on windward and leeward sites and are highly abundant at lower coastal elevations, and that conditions

allowing both mosquito and parasitic development of avian malaria are present at all elevations. Additionally, mosquitoes have been known to readily disperse within and across islands (Whiteman et al. 2005, Bataille et al. 2009a) and could easily disperse parasites and cause extinctions to highly susceptible avian populations residing at all elevational ranges in Galapagos, where all sites fall below the range of a stable disease-free refuge found in Hawaii. Thus, we recommend that further research be conducted to experimentally test the optimal conditions for parasitic and mosquito development within the climatic conditions in Galapagos. This, coupled with long-term mosquito sampling and fine-scale meteorological data collection, is needed to manage disease outbreaks and the transmission of parasites by arthropod vectors such as mosquitoes.

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Tables and Figures

Sites	1a	1b	2a	2b	3a	3b	4a	4b	5a	5b	6a	6b	7a	7b	8a	8b	9a	9b	Total
<i>A. taeniorhynchus</i>	23	73	0	1	110	0	0	0	20	5	21	48	4	4	4	110	205	129	757
<i>C. quinquefasciatus</i>	0	15	0	0	19	0	0	0	1	0	0	0	0	0	0	49	64	106	254
Elevation (masl)	13	15	221	300	311	328	351	384	618	595	391	381	253	267	170	167	48	16	
Site Name	Itabaca channel *		*		*		*		Los Gemelos		Santa Rosa		Bellavista		Miramar		Puerto Ayora		

Table 1: Total numbers of mosquitoes captured across 18 different sites and elevations on Santa Cruz. Asterisk represent sites on northern leeward facing slopes, Itabaca channel being the most northern site. Los Gemelos, Santa Rosa, Bellavista, Miramar and Puerto Ayora all occur on southern windward facing slopes

Coefficients	Estimate	p value	AIC, Degrees of Freedom (DF) and Residual Deviance
Rainfall	-0.039395	0.4848	AIC = 164.73 Residual deviance: 20.96 on 11 DF
Maximum temperature	-0.184778	0.4539	
Elevation	-0.004032	0.0856	
Minimum humidity	-0.130077	0.1965	
Maximum humidity	0.292703	0.0118	
Average DBH	0.006403	0.8163	
<hr/>			
Rainfall	-0.088998	0.1038	AIC = 161.2 Residual deviance: 20.919 on 12 DF
Maximum temperature	-0.348153	0.1139	
Elevation	-0.004502	0.0411	
Minimum humidity	-0.1474230	0.1190	
Maximum humidity	0.262139	0.0185	
<hr/>			
Rainfall	-0.079418	0.1216	AIC = 159.03 Residual deviance: 21.051 on 13 DF
Maximum temperature	-0.186733	0.2498	
Elevation	-0.004924	0.0183	
Maximum humidity	0.207343	0.0363	
<hr/>			
Rainfall	-0.057125	0.2687	AIC = 157.59 Residual deviance: 21.085 on 14 DF
Elevation	-0.004573	0.0293	
Maximum humidity	0.220127	0.0210	

Table 2: Backward stepwise regression with AICs and p values in selecting significant environmental factors for Negative Binomial GLM Model for *A. taeniorhynchus*. Covariates highlighted in bold were eliminated in subsequent analysis due to having the highest p value in model.

<i>A. taeniorhynchus</i> (n) ~ maximum humidity + elevation Residual deviance = 20.23 on 15 df AIC = 156.23 $\chi^2 = 0.139$				
	Estimate	Std. Error	Z value	Pr(> z)
Intercept	-19.563405	8.513575	-2.298	0.02157 *
Elevation	-0.005514	0.002085	-2.645	0.00817 **
Maximum humidity	0.251031	0.089108	2.817	0.00485 **

Table 3: Final Negative Binomial GLM Model with significant factors, AIC and goodness of fit test ($\chi^2 = 0.139$) for significant environmental factors influencing abundance of *A. taeniorhynchus* across sites. Asterisk represent significant codes 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1.

Coefficients	Estimate	p value	AIC, Degrees of Freedom (DF) and Residual Deviance
Maximum humidity	0.662710	0.0802	AIC = 22. 846 Residual deviance: 10.847 on 12 DF
Minimum humidity	0.004453	0.9860	
Elevation	-0.008692	0.1608	
Rainfall	-0.761149	0.5490	
Average DBH	-0.110474	0.6179	
<hr/>			
Maximum humidity	0.665093	0.0605	AIC = 20.847 Residual deviance: 10.847 on 13 DF
Elevation	-0.008701	0.1593	
Rainfall	-0.754803	0.5351	
Average DBH	-0.108820	0.5855	

Table 4: Backward stepwise regression with AICs and p values in selecting significant environmental factors to include in Generalized Linear Model for *C. quinquefasciatus*. Covariates highlighted in bold were eliminated in subsequent analysis due to having the highest p value in model.

<i>C. quinquefasciatus</i> ~ maximum humidity + elevation + rainfall				
Residual deviance = 11.34 on 14 df				
AIC = 19.359				
$\chi^2 = 0.657$				
	Estimate	Std. Error	Z value	Pr(> z)
Intercept	-61.408650	30.628773	-2.005	0.0450 *
Maximum humidity	0.654737	0.324059	2.020	0.0433 *
Elevation	-0.008854	0.005154	-1.718	0.0858 .
Rainfall	-0.476180	0.498957	-0.954	0.3399

Table 5: Generalized Linear Model with significant factors, AIC and goodness of fit test ($\chi^2 = 0.657$) for significant environmental factors influencing occurrence of *C. quinquefasciatus* across sites. Asterisk represent significant codes 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.

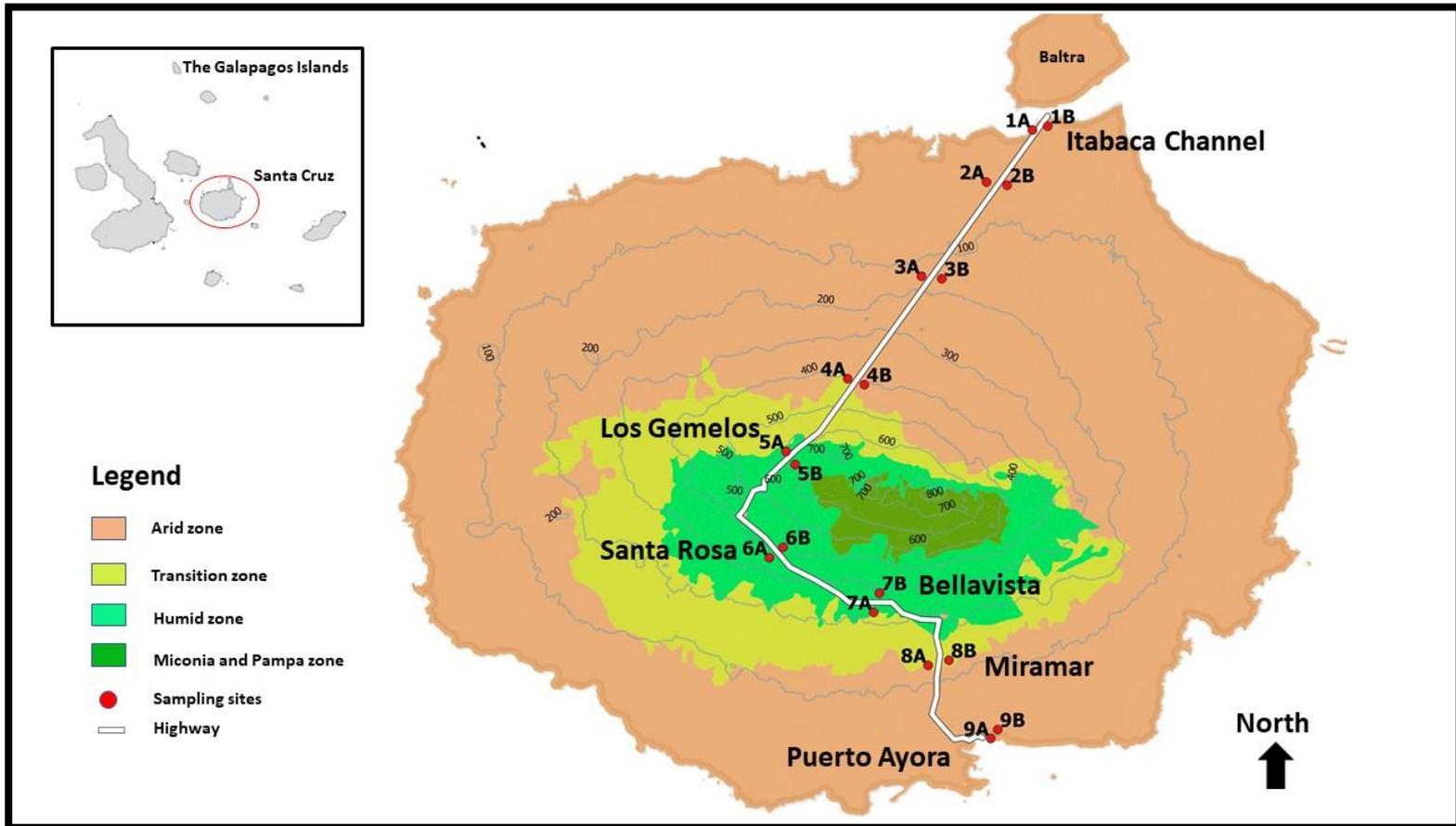


Figure 1: Map of Isla Santa Cruz with 100 m elevation contour lines. Map represents major vegetation zones and 18 sampling sites with accompanying names of sites such as Itabaca Channel, Los Gemelos, Santa Rosa, Bellavista, Miramar and Puerto Ayora in Isla Santa Cruz.

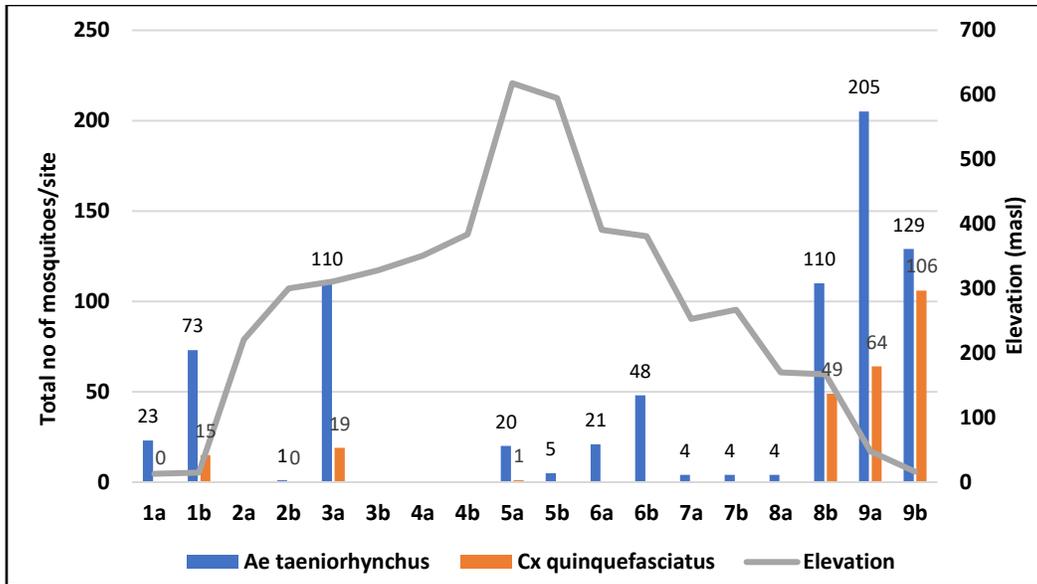


Figure 2: Total number of mosquitoes captured across 18 sites varying in elevation (masl) in Santa Cruz. Numbers above bar graph represent total number of mosquitoes representing *A. taeniorhynchus* and *C. quinquefasciatus* captured in 3 nights at each site.

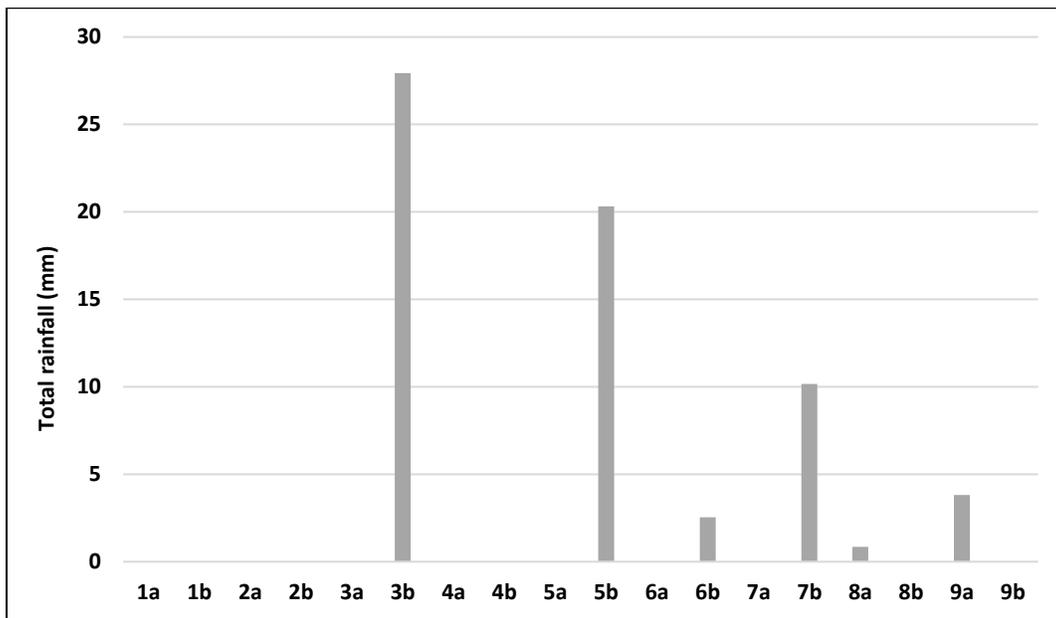


Figure 3: Total rainfall (mm) captured at each site during sampling period.

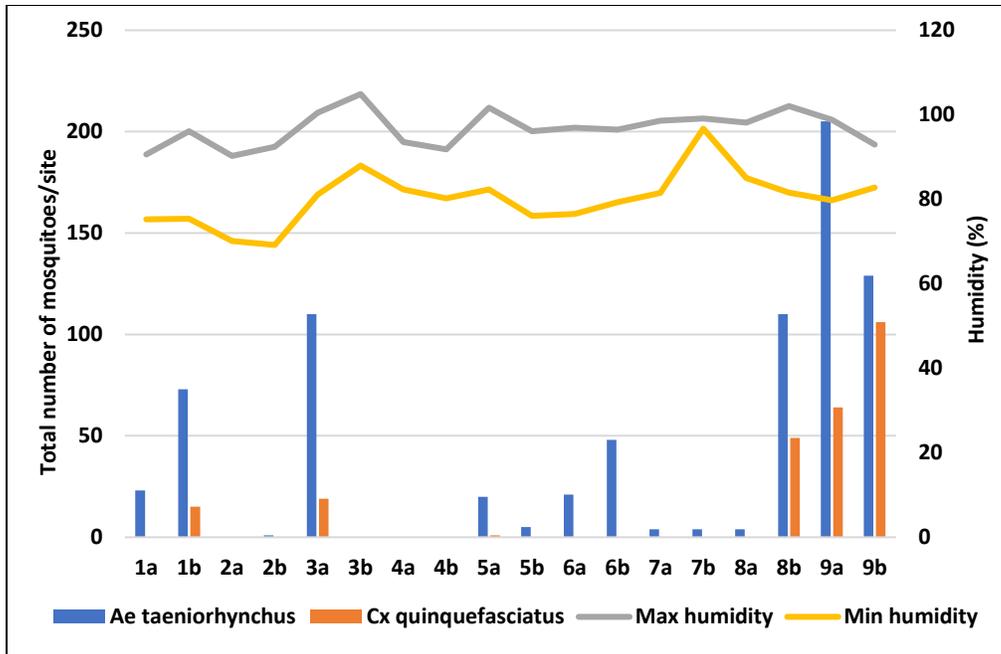


Figure 4: Average maximum and minimum humidity (%) recorded over 3 nights of trapping and the corresponding total numbers of *A. taeniorhynchus* and *C. quinquefasciatus* mosquitoes captured per site.

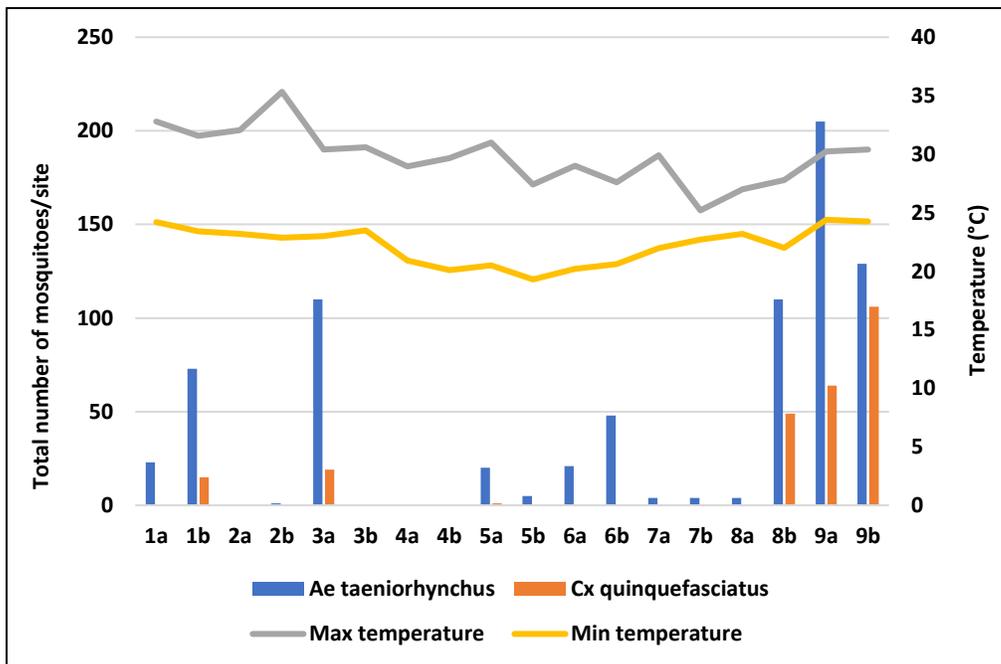


Figure 5: Average maximum and minimum temperature (°C) recorded over 3 nights of sampling with corresponding total numbers of *A. taeniorhynchus* and *C. quinquefasciatus* captured at each site.

Chapter 3

Title: Assessing the host feeding range of *C. quinquefasciatus* and *A. taeniorhynchus* on Isla

Santa Cruz, Galapagos

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Abstract

Bloodmeal host selection by mosquito vectors is an important component in understanding disease dynamics of pathogens that threaten endemic fauna in isolated islands such as Galapagos. Here, we use a combination of field techniques and PCR screening to identify vertebrate sources of mosquito bloodmeals. We sampled two mosquito species, *Aedes taeniorhynchus* and *Culex quinquefasciatus*, across 18 different sites in the summer of 2015 on Isla Santa Cruz, the second largest island in Galapagos, and the island with the largest human population. Mosquitoes were trapped using CDC light traps and CDC gravid traps and bloodmeal sources of engorged mosquitoes were identified by sequencing a portion of the vertebrate mitochondrial Cytochrome B gene. Our results show that out of 948 female mosquitoes captured, 301 PCR amplifications of bloodmeals were successful and showed that *A. taeniorhynchus* is a generalist feeder and feeds mainly on mammals, particularly humans, and *C. quinquefasciatus* is also highly anthropophilic on Santa Cruz. The high proportion of mammalian bloodmeals could represent locally available and abundant hosts on Santa Cruz. However, host surveys and estimates of relative abundances of vertebrate species will need to accompany mosquito trapping studies on non-inhabited and inhabited islands in Galapagos to further validate this.

Keywords: Mosquito, feeding patterns, Galapagos, *Aedes*, *Culex*, Santa Cruz

Introduction

Knowledge of blood-feeding preferences by mosquitoes can provide insight into disease dynamics and help manage parasites that pose threats to endemic wildlife. Many insects such as mosquitoes require a bloodmeal to complete their gonotrophic cycle and can thereby transmit bloodborne pathogens that threaten health of wildlife and humans (Bhatt et al., 2013; Greenwood et al., 2014; van Riper et al., 1986). Host preference by mosquitoes appears to be heritable (Gillies, 1964; Ulloa et al., 2004) but can also depend on ecological factors like host availability and abundance, vector abundance, habitat and climate (Simpson et al., 2012; Thiemann et al., 2011). In addition, when hosts become rare or limited, disease vectors may disperse to new habitats and modify their feeding behavior to a diverse range of hosts. This shift in feeding behavior by disease vectors may have serious implications for disease transmission and dynamics, especially in novel habitats. For instance, numerous endemic birds in Hawaii faced extinction from the co-introduction of avian malaria and avian pox, two virulent pathogens common to birds in continental areas. These parasites were likely carried to Hawaii through migratory birds from mainland continents (Atkinson and LaPointe, 2009). However, the introduction of *Culex quinquefasciatus* in the 1820s, brought to Hawaii in water casks in merchant ships from Mexico, helped transmit deadly pathogens from resistant migrants to naïve native birds, resulting in extinctions of many endemic Hawaiian bird species (van Riper et al., 1986; van Riper III et al., 2002).

The Galapagos Islands are volcanic in origin and situated almost 1000 km from the west coast of mainland Ecuador. The islands are known for their high endemism which inspired Charles Darwin's theory of evolution by natural selection (Darwin, 1859). Given its iconic natural system, its flora and fauna are well studied and human movements and impacts in the archipelago are at least partly controlled and monitored by the collective efforts of the

Galapagos National Park and the Charles Darwin Research Station. Despite these efforts, the archipelago already hosts arthropod vectors such as *C. quinquefasciatus* along with two other mosquitoes, the yellow fever mosquito (*Aedes aegypti*) and the black salt marsh mosquito (*Aedes taeniorhynchus*). Estimated to have naturally arrived ~200,000 years ago (Bataille et al., 2009b), *A. taeniorhynchus* oviposits in brackish water (Bataille et al., 2012). In contrast, *A. aegypti* and *C. quinquefasciatus* require fresh water for oviposition and have been estimated to have established populations in the archipelago in 2001 and 1985, respectively (Causton et al., 2006; Whiteman et al., 2005). *A. aegypti* is highly anthropophilic and has been found in human-inhabited zones such as Santa Cruz and Isabela (Asigau et al., 2017; Causton et al., 2006).

The black salt marsh mosquito, *A. taeniorhynchus*, has been shown to have a strong preference for taking bloodmeals from reptiles and mammals over birds in mosquitoes sampled on uninhabited islands in the Galapagos archipelago (Bataille et al. 2012). It is unknown how its feeding preferences may change on human-inhabited islands. In addition, the feeding preference and feeding range of a recent arrival, *C. quinquefasciatus*, in Galapagos remains unknown. Our knowledge of host-parasite associations in Galapagos also remains fragmentary; therefore, studies of feeding behavior by mosquitoes may provide clues to the arthropod vectors involved in disease transmission. One of these pathogens transmitted by mosquitoes includes the Haemosporidian blood parasite that causes avian malaria. Extensive sampling and molecular screening of endemic Galapagos penguin populations (*Spheniscus mendiculus*) revealed via PCR the presence of an avian parasite within the genus *Plasmodium* (lineage A) with infections detected in 3 – 9.4 percent of sampled penguins per year (Levin et al., 2013, 2009). However, the absence of gametocytes (stage of the parasite infective to arthropod vectors) within thin blood films prepared from infected penguins

suggests parasitic abortive development, or that penguins could be dead-end hosts. Three additional *Plasmodium* lineages (B, C, D) have since been discovered along with microscopic detection of a *Plasmodium* erythrocytic meront from a Cactus Finch (*Geospiza scandens*) and Haemosporidian trophozoites from a Vegetarian Finch (*Platyspiza crassirostris*) (Levin et al., 2013). Other additional pathogens known to infect Galapagos birds include several lineages of *Haemoproteus* (Order: Haemosporidia) (Levin et al., 2012, 2011; Padilla et al., 2004; Santiago-Alarcon et al., 2008), microfilariae (Merkel et al., 2007) and avian pox virus (Parker et al., 2011).

The transmission of pathogens in Galapagos may involve arthropod vectors such as mosquitoes. Therefore, it is important to understand the feeding range of mosquitoes. Here, we aimed to investigate the host feeding range of two mosquitoes common to Galapagos, *A. taeniorhynchus* and *C. quinquefasciatus*, and discuss their role in transmitting important pathogens that threaten endemic wildlife in Galapagos.

Methodology

Study Site

This study was conducted on Santa Cruz, which is part of the Galapagos archipelago. Consisting of 13 major islands and 19 smaller islands, the archipelago is volcanic in origin and predominantly arid and has high endemism and low biodiversity with 530 species of fish and 111 other vertebrate species of mammals, birds and reptiles. Terrestrial birds constitute 49 species of which 21 are endemic and 4 are endemic subspecies. There are 33 mammal species consisting of 2 endemic species and 29 reptilian species of which 20 are endemic (Swash and Still, 2005).

Our study was conducted on Isla Santa Cruz between May 20 - August 03, 2017. Santa Cruz is the second largest island in Galapagos with a land area of 986 km² and one of four inhabited islands along with Isabela, Floreana, and San Cristobal. Identified as having the largest human population among the islands, the 2010 census recorded 15,000 inhabitants on Santa Cruz, which is 60 percent of the archipelago's human population (INEC, 2010) and nearly double the population of the whole archipelago since 1998. Likewise, the tourism industry has dramatically increased in the latter half of the 20th century, especially among inhabited islands. In 1969, approximately 2000 people visited the Galapagos Islands, which is a small fraction of the 180,000 people who visited in 2012 (PNG, 2013). Compared to other islands, Santa Cruz hosts most of this human population and attracts tourists due to its developed infrastructure such as a hospital, schools, banks, shops, hotels and restaurants. Included in this infrastructure is a single 40 km paved road that extends from the north at Itabaca Channel, which is the entrance to Santa Cruz from the airstrip on adjacent Baltra Island, to the most southern tip at Puerto Ayora. Humans mainly inhabit the southern windward half of Santa Cruz since it provides ideal conditions for agriculture, and towns include Puerto Ayora, Miramar, Bellavista, Santa Rosa and Santa Martha.

Mosquito survey

We trapped mosquitoes across 18 trapping sites along the main highway that stretches from the north at Itabaca Channel to the south at Puerto Ayora. Using the highway as a transect, we established 9 trapping stations spaced 5 km apart and set two trapping locations spaced at 300 m at each station, totaling 18 independent trapping sites (See Figure 1). At each trapping site, we established a total of 4 points measuring 50m apart and alternated 2 CDC light traps (Model 512 John Hock Company, Gainesville, FL) and 2 CDC gravid traps (Model

1712 John Hock Company, Gainesville, FL) across these points. CDC light traps were baited with a CO₂ emitting concoction consisting of 250g sugar, 35g yeast and 2.5 liters of water to attract host-seeking mosquitoes (Gillies, 1980; Smallegange et al., 2010) and gravid traps were baited with a hay-yeast-water infusion to attract ovipositing mosquitoes (Reiter, 1986). Traps were set within one hour of dusk and mosquitoes were collected in the morning the next day. Mosquitoes were immobilized with chloroform, sexed, and identified to species level using morphological characters. We separated wild-caught mosquitoes into 2 groups: engorged or blood-fed mosquitoes and unfed mosquitoes, and classed them according to the Sella scale (1 = unfed; 2 – 6 = partial to full blood meal; 7 = gravid) (Detinova, 1962). Whole female mosquitoes were dissected into head/thorax and abdomen regions using sterile techniques and stored as single mosquitoes in Longmire's lysis buffer (Longmire et al., 1988) in preparation for subsequent DNA extraction and bloodmeal analysis. Whole male mosquitoes were preserved in 95 percent ethanol.

Bloodmeal analysis

Genomic DNA from engorged female mosquitoes was extracted using Machery Nagel NucleoSpin® Tissue Kit according to manufacturer instructions. We used a universal BM primer set developed by Kocher et al. (1989). This primer set specifically amplifies a fragment of 358 bp of the vertebrate Cytochrome B gene (Forward: 5'-CCC CTC AGA ATG ATA TTT GTC CTC A-3' and Reverse 5'-CCA TCC AAC ATC TCA GCA TGA TGA AA-3') in assessing sources of mosquito bloodmeals via Polymerase Chain Reaction (PCR) (Kocher et al., 1989). Negative controls were used (all reagents minus template DNA) and positive controls included different taxa representing wildlife DNA samples from Galapagos species. Positive controls consisted of two individuals of marine iguanas (*Amblyrhynchus*

cristatus), two species of birds (an introduced bird, the cattle egret (*Bubulcus ibis*) and an endemic bird, a large ground finch (*Geospiza magnirostris*), and finally, two samples from a mammal (*Homo sapiens*). The PCR reaction contained 15.875 μ L of sterile distilled water, 2.5 μ L of 10X buffer, 2 μ L of dNTPs, 1.5 μ L of MgCl₂, 1 μ L of each primer, 0.125 μ L of Taq and 1 μ L of extracted DNA template in producing a total volume of 25 μ L. Reactions were amplified through 36 cycles with the following parameters: 210 seconds at 95 °C (initial denaturation), 30 seconds at 95 °C (denaturation), 50 seconds at 60 °C (annealing), followed by a final extension step for 5 minutes at 72 °C (Hamer et al., 2009). Amplifications were assessed by gel electrophoresis using 1.5 percent agarose and positive PCR products were purified and sent to Eurofins Genomics LLC (12701 Plantside Drive, Louisville, KY 40299, USA) for sequencing.

Sequencing results were subjected to BLAST search in GenBank® (<http://www.ncbi.nlm.nih.gov>) and each chromatogram was inspected for sequence quality. Sequenced amplicons that yielded double or triple nucleotide peaks, likely representing bloodmeals from two or more vertebrate sources, were removed from analysis. Samples that produced an ambiguous amplicon with no match or with low quality peaks were re-run with a second reaction using an avian primer set (Forward: 5'-GAC TGT GAC AAA ATC CCN TTC CA-3' and Reverse: 5'-GGT CTT CAT CTY HGG YTT ACA AGA C-3) (Molaei et al., 2006). This primer set targets a 508 bp fragment size in the Cytochrome B gene under reaction conditions described above (Hamer et al., 2009; Molaei et al., 2006). If amplicons failed to produce high quality, single peaks, we further subjected samples to a third reaction targeting 772 bp in the mammalian Cytochrome B gene (primers 5'-Forward: CGA AGC TTG ATA TGA AAA ACC ATC GTT G-3' and 5'-TGT AGT TRT CWG GGT CHC CTA-3') (Molaei et al., 2006). Reactions also followed the same conditions described above.

Samples that produced single peaks in any of the three reactions with a satisfactory match of 98 – 100 percent to sequences in GenBank were accepted as the source of origin for mosquito bloodmeals.

Results

Mosquito Survey

A total of 1,011 mosquitoes were collected in the summer of 2015 over 216 trap nights and consisted of 757 *A. taeniorhynchus* and 254 *C. quinquefasciatus*. We collected 38 males and 719 female *A. taeniorhynchus* (Table 1) and 26 males and 228 females of *C. quinquefasciatus* (Table 2). Female *A. taeniorhynchus* were captured at all but four sites on Santa Cruz. Abundances of female *A. taeniorhynchus* were highest in coastal elevations and generally declined with elevation; 30 percent of female mosquitoes were captured in Puerto Ayora (9a and 9b), 15 percent in Miramar (8a and 8b) and site 3a, and 12 percent in Itabaca Channel (Table 1). In contrast, *C. quinquefasciatus* female mosquitoes were captured at only 6 sites on Santa Cruz with 67 percent of captures occurring in Puerto Ayora (9a and 9b) and 21 percent at site 8a at Miramar (Table 2).

Bloodmeal analysis

For 719 female *A. taeniorhynchus* mosquitoes, molecular screening identified 242 females as positive for taking a bloodmeal from a vertebrate host. Of these, 232 *A. taeniorhynchus* bloodmeals were resolved with sequencing chromatograms showing single high-quality peaks. Ten bloodmeal sources remained unresolved and either failed to amplify even after multiple PCR attempts or remained ambiguous with double peaked sequences (Table 1). We identified 95 percent (220 mosquitoes) of bloodmeal sources from humans

(*Homo sapiens*), 2 percent (5 mosquitoes) from cattle (*Bos taurus*) and 1.7 percent (4 mosquitoes) from Galapagos tortoises (*Chelonoidis* spp) (Figure 2). A bloodmeal from one mosquito captured at site 6B on Santa Rosa (381 masl) included a bird belonging to the family Hirundinidae and a 100 percent match to *Tachycineta bicolor*. Another *Aedes* mosquito captured at site 1B on Itabaca Channel was identified to have taken a bloodmeal from a reptile (Class: Reptilia, Order: Squamata). A bloodmeal from one *Aedes* mosquito captured at site 1B on Itabaca Channel was identified as having fed from a mammal in the order Chiroptera (bats) (Figure 2). Humans were detected as a source of bloodmeal in mosquitoes captured both in southern and northern Santa Cruz and at low and high elevations. Puerto Ayora (site 9B) was the site where we found the highest number of mammalian bloodmeals and totaled 91 mosquitoes detected with human bloodmeals. Mosquitoes with humans as a source of bloodmeals were captured at elevations of ~ 300 masl and at the highest elevation sites such as in Los Gemelos (site 5A, 618 masl). Cattle (*Bos taurus*) as a source of bloodmeals were identified in 4 mosquitoes captured in Santa Rosa (site 6A and 6B) and in one mosquito captured at site 9B in Puerto Ayora. All mosquitoes identified with bloodmeals from Galapagos tortoises (*Chelonoidis* spp.) were captured at site 9B in Puerto Ayora (Figure 2); there is a captive breeding program for tortoises at the Park headquarters located just outside of Puerto Ayora.

For a total of 231 female *Culex* mosquitoes captured, molecular screening identified 75 mosquitoes with bloodmeals. Of these, 69 mosquitoes had bloodmeals that were resolved with chromatograms showing single high-quality peaks, thus indicating a single source of bloodmeal from a vertebrate species (Table 2). A total of 68 out of 69 of these bloodmeals were identified as human with 88 percent (n = 60) of blood-fed mosquitoes captured in Puerto Ayora (site 9A and 9B) alone (Figure 3). We identified a single human-fed *Culex* mosquito at

site 8B in Miramar, located 5 km north of Puerto Ayora and at site 6B, located at Santa Rosa. Mosquitoes identified with human bloodmeals were also captured in northern sites 3A and on the most northern site at Itabaca Channel (site 1A). One mosquito captured at site 6B was identified as positive for having a bloodmeal from a bird belonging to the *Hirundinidae* family with a 100 percent match to *Tachycineta bicolor*.

Discussion

Our analysis of the blood-feeding behavior of mosquitoes gives insight into their roles as disease-carrying vectors on an inhabited island in Galapagos. We found that both *A. taeniorhynchus* and *C. quinquefasciatus* are widespread and that sites with highest abundances of engorged female mosquitoes are those that record high mosquito abundances in general. The number of bloodmeals of *A. taeniorhynchus* was three times that of *C. quinquefasciatus* and this corresponded to the sample size of female mosquitoes of each species collected in the summer of 2015. Since we sampled in the dry season of 2015, it is not surprising that we captured low abundances of *C. quinquefasciatus*, a species whose females require fresh water to oviposit eggs. On the other hand, since *Aedes taeniorhynchus* females oviposit in brackish water, their high abundances could be attributed to the availability of mangrove habitats as well as ideal environmental conditions conducive for mosquito breeding (Asigau and Parker, 2018).

Aedes taeniorhynchus has been shown to feed primarily on mammals and reptiles in Galapagos (Bataille et al., 2012). Our study supports this finding with 99 percent of bloodmeals identified from mammalian and reptilian hosts and included humans, bats, cattle, land tortoises and lava lizards. The only non-reptilian/non-mammalian bloodmeal was identified as *Tachycineta bicolor* (tree swallow) which could be a vagrant in Galapagos. The

mosquito bloodmeal could also be from other birds in the *Hirundinidae* family such as the endemic Galapagos martin (*Progne modesta*), which is found in the highlands of central and southern islands of the archipelago or the purple martin, *Progne subis*, an infrequent visitor.

This provides support to the finding that *A. taeniorhynchus* mosquitoes in Galapagos prefer mammals and reptiles over birds (Bataille et al., 2012). Mammalian bloodmeals were highest in our study with 95 percent of engorged *Aedes* females identified as having fed from mammals. Mammal bloodmeals were found across the island of Santa Cruz indicating that this feeding behavior is widespread. In areas with human settlement such as in Puerto Ayora, Miramar and Santa Rosa, numbers of engorged mosquitoes were highest, indicating humans as an important source of bloodmeals for mosquitoes. We also found a high proportion of human bloodmeals in mosquitoes captured at Itabaca Channel, which is the point of entrance for tourists or visitors to Santa Cruz and Galapagos. Both *A. taeniorhynchus* and *C. quinquefasciatus* feed primarily at night and our night-time trapping protocol allowed us to sample when humans were less active and mosquito blood-feeding habits were at its peak. Since the majority of bloodmeals originated from humans in our study, we also did not need to include any foraging ratio analysis. However, we do recommend that future sampling of mosquitoes and vertebrate hosts be conducted during diurnal periods as well to better quantify host abundance and determine mosquito preference by use of the foraging ratio analysis (Kent, 2009), which estimates the significance of host bloodmeal preference as a function of relative abundance of different host species.

We also captured engorged mosquitoes of *A. taeniorhynchus* and *C. quinquefasciatus* with bloodmeals at uninhabited sites, Los Gemelos (site 5A) and site 3A, suggesting dispersal or movement of mosquitoes throughout the island of Santa Cruz. Mosquitoes have been known to disperse between and within islands in Galapagos through human-aided

transportation such as airplanes and boats (Bataille et al., 2009a) and the availability of a well-developed road network in Santa Cruz could further facilitate the movement of mosquitoes. *Aedes taeniorhynchus* is known to disperse up to 40 km (Provost, 1957) while *C. quinquefasciatus* can travel up to 3km in distance (Lapointe, 2008; Medeiros et al., 2017; Reisen et al., 1991) and their long-range dispersal could further broaden the range of wildlife pathogens.

Adults of female *A. taeniorhynchus* feed primarily at night and are hematophagous or blood-feeders, while males may nectar-feed (Burkett-Cadena 2013). Female mosquitoes utilize blood from vertebrate species to develop their eggs; however, this species is partially autogenous, meaning that it can oviposit an initial batch of eggs without a bloodmeal (Lea and Lum, 1959). Even though a bloodmeal is not a pre-requisite for egg production in *A. taeniorhynchus*, autogenous females readily consume a bloodmeal during the first and second day following emergence and blood-feeding can significantly increase egg production (O'Meara and Evans, 1973). Abundant vertebrate species such as mammals and reptiles in Galapagos may therefore provide a readily available foraging resource for partially autogenous *A. taeniorhynchus* female mosquitoes in producing a large initial egg batch.

Examination of blood-fed mosquitoes in our study showed an almost exclusively mammalian diet of *Culex quinquefasciatus* in Santa Cruz. With the exception of one bloodmeal from a bird belonging to the Hirundinidae family, all analyzed bloodmeals were identified as human. These findings are consistent with research that indicate that this species is both highly anthropophilic (Mboera and Takken, 1999; Samuel et al., 2004), an inherent opportunistic feeder (Takken and Verhulst, 2013), and a generalist feeder, meaning that it feeds indiscriminately on both birds and mammals (Zinser et al., 2004). Our findings may also indicate humans as one of the most abundant host species that is locally available, but

this does not necessarily mean that it is the preferred host. For instance, bloodmeal screening from *C. quinquefasciatus* captured in Kenya revealed only 3 to 9.8 percent of human bloodmeals; the majority of bloodmeals originated from other mammals such as cattle, goats and donkeys (Muturi et al., 2008). In Tanzania, experimentation with an equal availability of three vertebrate species found *C. quinquefasciatus* behavior highly anthropophilic (Mboera and Takken, 1999). In other sites, *Culex quinquefasciatus* has also been shown to generally prefer feeding on birds (Zinser et al., 2004) and occasionally on reptiles, amphibians, and mammals (Farajollahi et al., 2011; Janssen et al., 2015). In northeastern Mexico, foraging ratios of *C. quinquefasciatus* were highest for chickens compared to humans, horses and pigs and this was attributed to chickens being highly abundant in the area of study. In Galapagos, the agricultural zone includes Bellavista and Santa Rosa, however, our mosquito traps were placed more closely to human settlements than agricultural sites and therefore could have resulted in the detection of human bloodmeals than from farm animals such as chickens, pigs and cows. Nevertheless, the high plasticity in feeding behavior in *C. quinquefasciatus* indicates that even though it is an inherent opportunistic feeder, its feeding behavior varies with locally available and abundant species. Thus, it is not surprising that we identified a high proportion of human bloodmeals from mosquitoes captured in human-inhabited sites such as Puerto Ayora, Miramar, and Itabaca Channel.

The high proportion of mammalian bloodmeals in the inhabited island of Santa Cruz could give us clues to the transmission of wildlife pathogens among hosts. For instance, if mosquitoes, being highly opportunistic, feed more frequently on highly abundant and locally available non-avian host species, the chances of detecting avian parasites is small. In addition, the avian malaria parasite (*Plasmodium* spp.) has a very low infection rate in Galapagos and may be difficult to detect, particularly if competent vectors such as *C. quinquefasciatus* are

not abundant and are feeding mostly on mammalian or reptilian hosts. For instance, *Culex* mosquitoes modify their feeding preferences based on host availability and abundance and provide a bridge in the transmission of West Nile Virus from birds to humans (Hamer et al., 2009; Molaei et al., 2006). A detailed study integrating feeding behavior of mosquitoes and composition of host species showed that American robins, which are competent WNV hosts, were preferentially fed on by a closely related mosquito species, *C. tarsalis*. However, during periods of robin dispersal and migration, *C. tarsalis* shifted its feeding preferences from birds to humans. This greatly amplified number of human infections, particularly when mosquito infection prevalence was high from feeding on infected robins (Kilpatrick et al., 2006). *Culex quinquefasciatus* has the capacity to transmit avian malaria (van Riper et al., 1986) but the low malarial infection rate and generalist feeding behavior of *Culex* could be minimizing the chances of detection of *Plasmodium* in Galapagos mosquito sampling. Additional studies investigating the feeding preferences of mosquitoes on islands without human populations along with experimental infection of hosts and arthropod vectors are recommended to resolve this question. Our study highlights the importance of determining the host feeding range of mosquitoes and their feeding preferences in understanding the disease dynamics of wildlife pathogens such as avian malaria. This knowledge is important towards managing pathogens that may threaten the conservation of endemic wildlife, particularly avifauna in isolated islands such as Galapagos.

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Galápagos Islands Establishment of the avian disease vector *Culex quinquefasciatus* Say, 1823 (Diptera: Culicidae) on the Galápagos Islands, Ecuador. *Ibis Br. Ornithol. Union* 147, 844–847.

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Tables and Figures

Figure 1: Map of 18 mosquito sampling sites extending from the most northern site, Itabaca Channel to the most southern site, Puerto Ayora. Names of localities (Itabaca Channel, Los Gemelos, Santa Rosa, Bellavista, Miramar and Puerto Ayora) are also indicated beside their corresponding mosquito sampling sites.

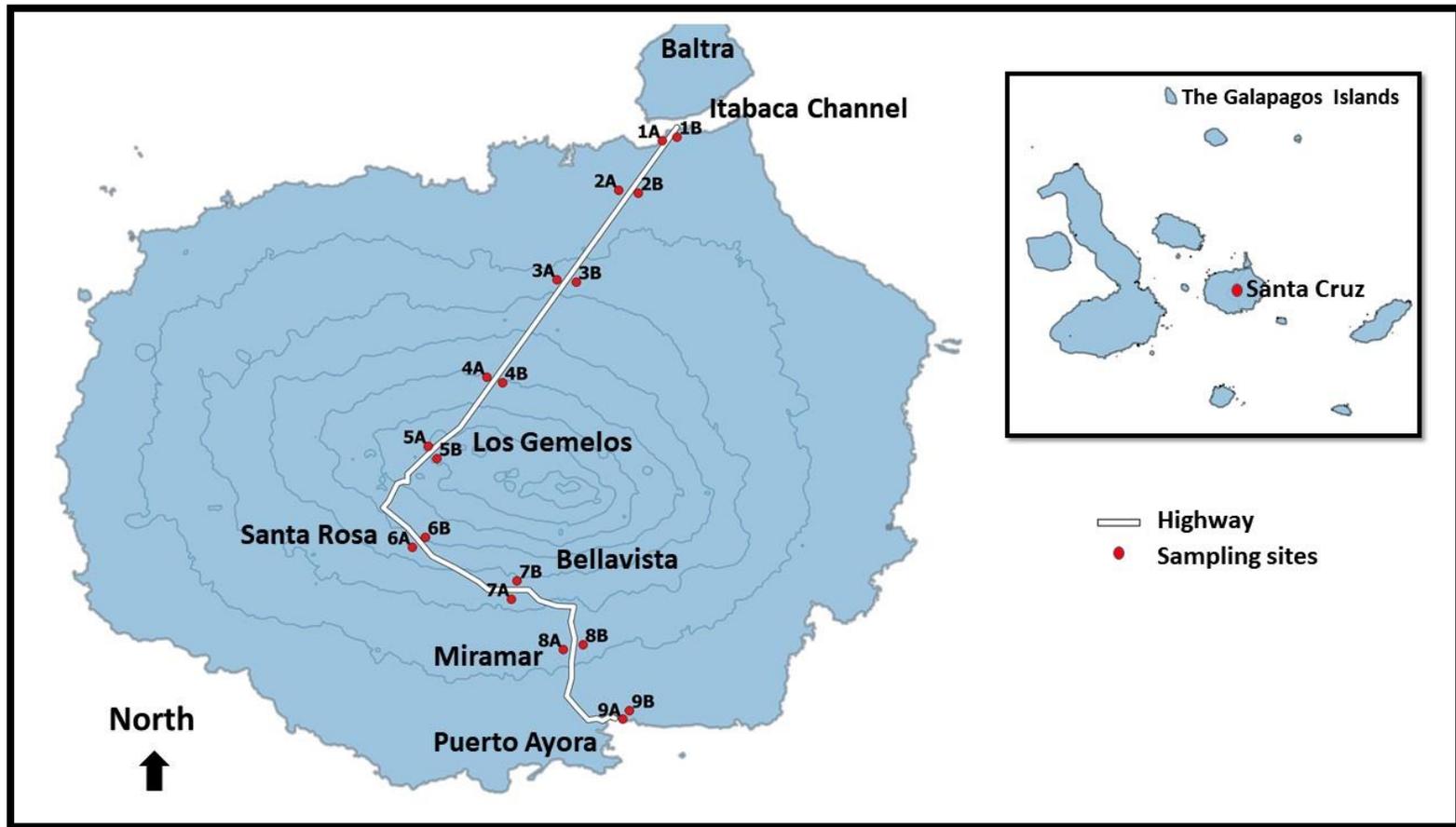


Figure 2: Host and site feeding range of *Aedes taeniorhynchus*. Numbers indicated in bars represent counts of resolved bloodmeals and numbers in yellow bars represent counts of unresolved/ambiguous sequences. *Homo sapiens*, *Bos taurus* and *Chiroptera* are mammalian families. *Chelonoidis* and *Acanthodactylus* represent reptilian families and *Hirundinidae* represents an avian family.

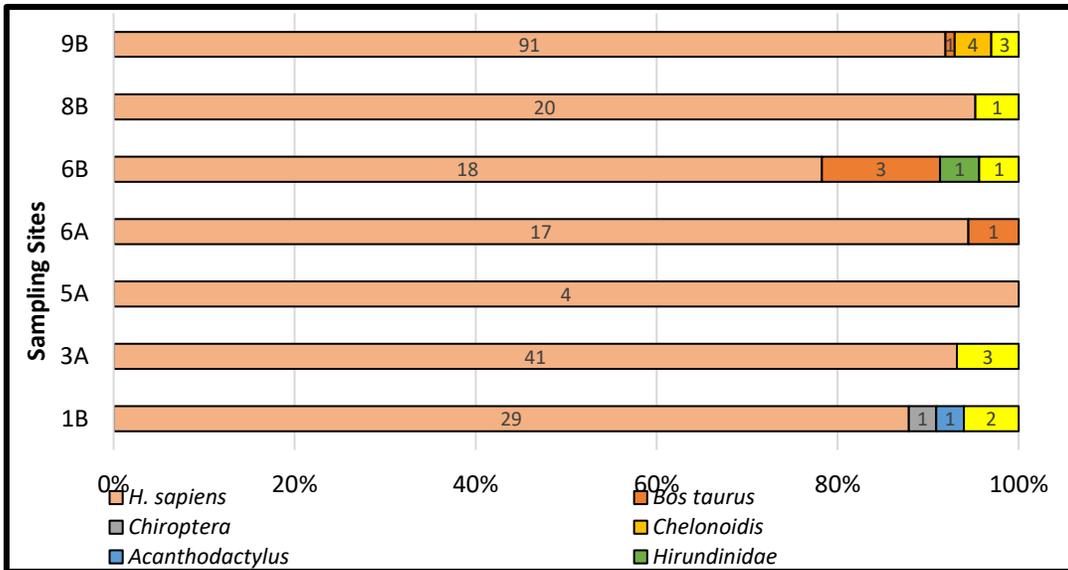
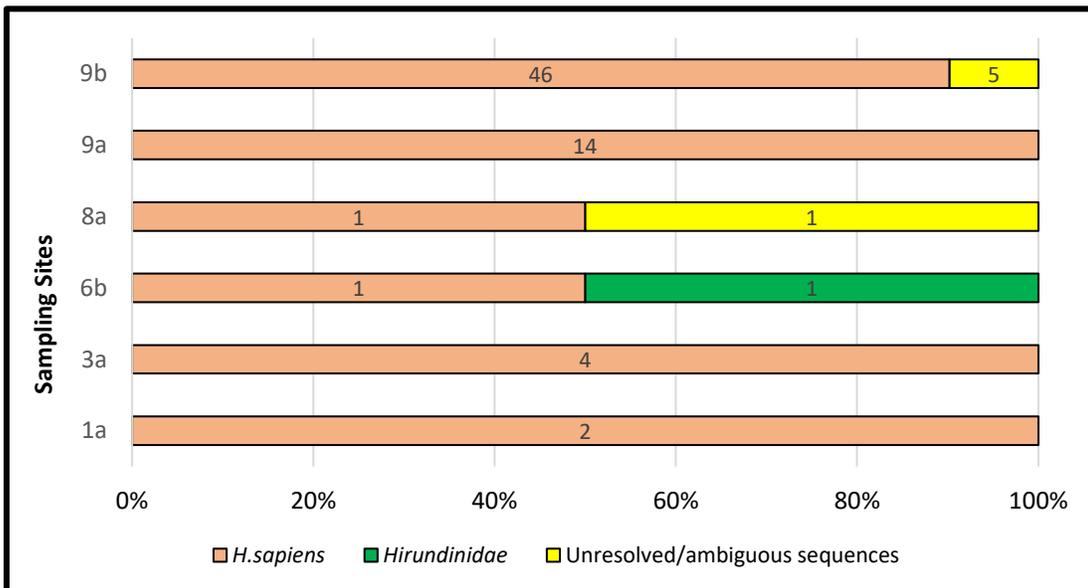


Figure 3: Host and site feeding range of *Culex quinquefasciatus*. Numbers indicated in bars represent counts of resolved bloodmeals and numbers in yellow bars represent counts of unresolved/ambiguous sequences.



Sites	Total male captured	Total female captured	Total engorged mosquitoes	Total resolved bloodmeals
1a	0	23	0	0
1b	2	71	33	31
2a	0	0	0	0
2b	0	1	0	0
3a	5	105	44	41
3b	0	0	0	0
4a	0	0	0	0
4b	0	0	0	0
5a	2	18	4	4
5b	0	5	0	0
6a	0	21	18	18
6b	1	47	23	22
7a	0	4	0	0
7b	0	4	0	0
8a	2	2	0	0
8b	1	109	21	20
9a	3	202	0	0
9b	22	107	99	96
TOTALS	38	719	242	232

Table 1: Total numbers of female and male mosquitoes belonging to *Aedes taeniorhynchus* captured across 18 sites in Santa Cruz. Included are the total number of engorged mosquitoes captured and resolved bloodmeals from female mosquitoes.

Sites	Total male captured	Total female captured	Total engorged mosquitoes	Total resolved bloodmeals
1a	0	3	3	2
1b	4	11	0	0
2a	0	0	0	0
2b	0	0	0	0
3a	5	14	4	4
3b	0	0	0	0
4a	0	0	0	0
4b	0	0	0	0
5a	0	1	0	0
5b	0	0	0	0
6a	0	0	0	0
6b	0	0	2	2
7a	0	0	0	0
7b	0	0	0	0
8a	0	0	2	1
8b	0	49	0	0
9a	12	52	15	14
9b	5	101	49	46
TOTALS	26	228	75	69

Table 2: Total numbers of female and male mosquitoes belonging to *Culex quinquefasciatus* captured across 18 sites in Santa Cruz. Included are the total number of engorged mosquitoes captured and resolved bloodmeals from female mosquito

Chapter 4

Disease screening of mosquitoes in the Galapagos Islands

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Abstract

An avian malaria parasite (Lineage A in the genus *Plasmodium*) has been recently identified frequently in endemic Galapagos penguins, *Spheniscus mendiculus* and less frequently in passerines in the Galapagos islands. With the objective of understanding the arthropod vector's role in transmitting this malarial parasite, we collected mosquito species *Aedes taeniorhynchus*, *Culex quinquefasciatus* and *Aedes aegypti* across three different islands and several elevation sites from 2012 to 2015. Field captured mosquitoes were screened for avian malaria parasites using molecular techniques. We also inspected microscopic slides of mosquito salivary glands to search for sporozoites, the stage of the parasite infective to the vertebrate host. We captured a total of 15,125 mosquitoes and screened 13,692 female mosquitoes for avian malaria parasites. We identified a pool of 5 *A. taeniorhynchus* positive with *Plasmodium* Lineage A avian malaria parasite from abdomens of mosquitoes captured in Puerto Vilamil, Isabela in 2013. However, microscopic evaluations of prepared salivary gland smears from those mosquitoes identified no sporozoites. We also identified *Haemoproteus multipigmentatus* in abdomens of 5 individual *A. taeniorhynchus* captured in Puerto Ayora in Santa Cruz in 2015. The non-detection of sporozoites in salivary glands and identification of parasites in abdominal regions of mosquitoes suggests the feeding of arthropods on infected birds, but not the involvement of those mosquitoes in a transmission pathway. However, this result does not reveal the vector competence of mosquito species in transmitting parasites of genera *Plasmodium* and *Haemoproteus*. Experimental infection of mosquitoes will need to be conducted to understand vector competence of arthropod species and their role in the disease dynamics of avian malaria in Galapagos.

Keywords: Plasmodium, Haemoproteus, Aedes, Culex, Galapagos

Introduction

High in endemism and known as hotspots for generating biodiversity, isolated oceanic islands contain nine times more endemic vertebrate species than mainland regions of the same size (Kier et al. 2009). However, their isolation renders their wildlife vulnerable to introduced parasites and pathogens, since island species have evolved in the absence of many diseases. Their low genetic diversity and limited ability to genetically adapt to environmental change such as global climate change, diseases, introduced predators and competitors and land degradation can be detrimental and result in extinction of species. An example of this effect was demonstrated in Hawaii in the 1800s, where the co-introduction of avian malaria, avian pox and the Southern House mosquito, *Culex quinquefasciatus* resulted in the extinction of several species of endemic birds belonging to the Family Drepanididae (Warner 1968). Avian malaria is a disease caused by a protist belonging to the family Haemosporida and genus *Plasmodium* which contains different lineages that infect mammals, birds, and reptiles. It is a mosquito-borne parasite whose pathogenic effects can result in devastating consequences once introduced to isolated islands as in Hawaii.

The Galapagos archipelago forms a group of isolated islands consisting of 13 major islands, numerous satellite islands and smaller islets situated 1000km west of the coast of Ecuador. These islands host high endemism of flora and fauna species that inspired Charles Darwin's theory of evolution by natural selection (Darwin 1839). Due to its iconic status, the islands are highly protected and monitored by the Galapagos National Park and Charles Darwin Research Center. However, long term wildlife disease surveying efforts revealed an avian blood parasite within the genus *Plasmodium* (lineage A) found within the endemic Galapagos penguin, *Spheniscus mendiculus*. Prevalence of this parasite was found to range from 3 to 9.4 percent in

infected penguins sampled across six field seasons from 2003 – 2009 (Levin et al. 2009, 2013). Microscopic evaluations of blood smears prepared from infected individuals revealed no gametocytes, the stage of the parasite infective to the host and thereby suggesting abortive development of the parasite within the penguin. Three other distinct lineages (lineage B, C and D) have since been discovered following further sampling of passerines along major shorelines in Galapagos. However, these lineages were identified only once in sampling years suggesting their sporadic presence on the islands. In addition to its detection in penguins in multiple years on multiple islands, Lineage A was detected in one Medium Ground (*Geospiza fortis*) finch from Santa Cruz in 2005 and in Yellow Warblers (*Dendroica petechia aureola*) sampled near Puerto Vilamil in 2008. Similar to infected penguins, PCR positive individuals lack gametocytes suggesting abortive development in these species. Since Lineage A of the Plasmodium parasite was identified in several species sampled across different islands and in different years, it is suggested that it is transmitted regularly and established on the islands (Levin et al. 2009, 2013). However, the competent avian hosts responsible for maintaining the transmission cycle of Lineage A malarial parasites remains unknown, as does the identity of the arthropod vector.

There are currently three mosquito species capable of transmitting wildlife parasites. *Aedes taeniorhynchus* is a widely distributed mosquito in the archipelago and naturally arrived ~200,000 years ago (Bataille et al. 2009b). Commonly known as the black salt marsh mosquito, female mosquitoes are known to oviposit in brackish water such as mangroves and salt marshes along the coast (Provost 1951). Interestingly, the same lineage of *Plasmodium* infecting the endemic Galapagos penguin (*Spheniscus mendiculus*) was also detected via PCR in abdomen and head/throat regions of *A. taeniorhynchus* from Socorro Island in Mexico (Carlson et al. 2011). A recent arrival, *Culex quinquefasciatus*, which was first documented in 1985 (Whiteman et al. 2005), is highly anthropophilic, oviposits in fresh water, and has been associated with the

transmission of avian malaria (Warner 1968, van Riper et al. 1986). A likely vector of *Avipoxvirus* in Hawaii (LaPointe et al. 2005), Galapagos individuals also prove to be competent vectors for West Nile Virus under experimental conditions (Eastwood et al. 2011) and suspected mechanical vectors for *Avipoxvirus* (Thiel et al. 2005). Since both *A. taeniorhynchus* and *C. quinquefasciatus* are known transmitters of avian malaria, it is possible that either species could be carrying these parasites amongst endemic wildlife hosts. The most recent of arrivals, *Aedes aegypti*, also known as the yellow fever mosquito, is highly anthropophilic and found on the islands of Santa Cruz, Isabela and San Cristobal (Causton et al. 2006, Asigau et al. 2017). This species, like *C. quinquefasciatus*, also favors fresh water habitats for female mosquitoes to oviposit and is not known to transmit avian malaria.

The presence of both avian malarial parasites and arthropod hosts warrants the need to understand the disease dynamics of avian malaria in Galapagos. With specific focus on arthropod hosts, we aimed to identify possible vectors of Plasmodium in Galapagos and screen for other disease agents transmissible by mosquitoes. Using a combination of field techniques, molecular screening and microscopy, our main goal was to identify the mosquito's potential in being a competent vector of avian malaria and assess their geographical distribution across the Galapagos archipelago. This is a necessary first step towards managing wildlife diseases that threaten the conservation of endemic species in the archipelago.

Methodology

Study Site

We conducted this study on three major islands in Galapagos; two inhabited islands, Isabela and Santa Cruz and the uninhabited island of Santiago (Figure 1). Santa Cruz is the second largest island with a land area of 986 km² and hosts the largest human population among

the islands, recording 15,000 local inhabitants. Isabela, also an inhabited island, is the largest island in Galapagos. Isla Isabela has a land area of 4641 km² and holds the highest peak of 1707 m which is found on Volcan Wolf. The uninhabited island of Santiago holds two overlapping inactive volcanoes, has a land area of 585 km² and a maximum altitude of 907 m.

We collected mosquitoes during four field seasons; from May 26 to July 5, 2012, June 23 to August 1, 2013, February 6 to June 7, 2014 and May 20 to August 03, 2015. In 2012, we sampled on southern Isabela and the island of Santa Cruz and, in 2013 and 2014, we included an additional island, Santiago into our sampling regime (Figure 1). We established three sites on Isabela at sea level, Puerto Vilamil - 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, three sites were established at Puerto Ayora - 0m ASL (S 00° 44' 35.5", W 090° 18' 09.4"); Bellavista - 180m ASL (S 0° 41' 42.3", W 90° 19' 36.9" and Media Luna - 500m ASL (S 00° 39' 58.9", W 90° 19' 30.3"). In Santiago, we established two sites; 0m ASL (S 00°14' 42.50", W 90° 52' 7.75") and 180 meters ASL (S 00° 11' 39.4", W 90° 49' 25.3"). For the fourth field season in 2015, we established 9 (Station 1 – 9) trapping stations spaced at 5 km at equal distance apart along the main highway in Santa Cruz. For each trapping station, we established two replicates measuring 300m apart and totaling 18 trapping sites (Figure 2).

Mosquito Trapping

In field seasons 2012 to 2014, we established 4 – 8 mosquito traps consisting of an equal number of CDC light traps (Model 512 John Hock Company, Gainesville, FL) and a CDC gravid traps (Model 1712 John Hock Company, Gainesville, FL) and trapped at each site once per field

season for three to six consecutive nights. Light traps, which attract host-seeking mosquitoes (Onyango et al. 2013), were baited with a concoction of sugar/yeast/water mixture (250g/35g/2.5L) to emit CO² (Smallegange et al. 2010). Gravid traps were baited with a hay/yeast/water infusion to attract ovipositing mosquitoes and ideally infected female mosquitoes that have taken a bloodmeal. In 2015, we sampled at each of the 18 sites once for three consecutive nights with 2 CDC light traps and 2 CDC gravid traps. For all field seasons, we set traps one hour before dusk (~6:00pm), and collected mosquitoes the next morning. Wild captured mosquitoes were immobilized with chloroform, sexed and identified to 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 mosquitoes were immobilized with chloroform, we dissected as many females as time allowed before desiccation. We prepared salivary gland smears according to the standard protocol (Valkiūnas 2005). We applied sterile techniques before each dissection by using a clean slide and dipping dissection tools into 10 percent bleach, rinsing in distilled water, drying and applying heat to tools using a Bunsen burner to avoid cross-contamination of specimen DNA. Salivary gland smears were fixed with methanol immediately after drying and were stained with Giemsa (4mL stock Giemsa/1L phosphate buffer) within 2 – 3 weeks of fixing according to standard protocol (Valkiūnas 2005). For each salivary gland preparation, we separated female mosquito abdomens from heads and thorax regions and pooled separately in 180µL of Longmire's lysis buffer. Pool sizes ranged from 1 – 9 individuals and included mosquitoes collected by date from a single site and trap type. Engorged female mosquitoes were

preserved individually in 180 μ L for preservation of bloodmeals. Mosquitoes not dissected were stored whole in 95 percent ethanol at -20 degrees Celsius for later processing and DNA extraction in the laboratory at the University of Missouri – St. Louis. Ethanol preserved samples were further dissected into head and abdomen/thorax regions using sterile techniques and were air dried in a fume hood from three hours to overnight depending on the pool size. This method allowed for the ethanol to evaporate from preserved mosquitoes prior to DNA extraction.

DNA Extraction, PCR, and Sequencing

Dried tissues of mosquitoes were first homogenized with a heat-sealed pipette tip and genomic DNA from engorged female mosquitoes was extracted using Machery Nagel NucleoSpin® Tissue Kit according to manufacturer instructions. All extracted samples containing abdomen and head/thorax regions were subject to screening for Plasmodium via a nested PCR reaction. Primer sets HAEMF/HAEMR (initial) and HAEMNF/HAEMNR2 (nested) target a 580bp fragment of the parasite's mitochondrial b gene (cty b) according to conditions developed by Waldenström et al. (2004). For each reaction, we used a positive control consisting of a PCR positive DNA sample from an infected Galapagos penguin (*Spheniscus mendiculus*) and a negative control containing only PCR reagents but no mosquito DNA. Amplicons were run on a 1.5 percent agarose gel at 90 V for an hour with GelStar (Lonza, Rockland, ME).

We identified positive samples by a 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) and were sequenced using inner reaction 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. We manually edited forward and reverse sequences before assembling consensus sequences using SeqMan 4.0 software (Lasergene, DNASTAR, Inc, Madison, Wisconsin). For samples that we could not sequence using the ABI 3130 Genetic Analyzer due to time constraints, we sent to Eurofins Genomics LLC (12701 Plantside Drive, Louisville, KY 40299, USA) for sequencing. Sequencing results were subjected to BLAST search in GenBank® (<http://www.ncbi.nlm.nih.gov>) and each chromatogram was inspected for sequence quality.

Microscopy

We performed microscopic evaluations of slides prepared to detect Plasmodium sporozoites (i.e.: the life stage of the parasite infective to vertebrate host). Microscopy accompanying molecular screening of diseases is a comprehensive tool to investigate parasitic infections of the mosquito vectors. We examined whole salivary gland smears using either an Olympus BH-2 or Olympus CX31 (Olympus, Shinjuku, Tokyo, Japan) with 1000X total magnification to identify the presence of the sporozoites. We report results of slide readings for 2012 only. Time taken to read all fields of a salivary gland preparation varied from ~30-60 minutes, depending on the size of the preparation and the amount of artifact present.

Results

Mosquito Survey

In 2012, we captured a total of 2974 *C. quinquefasciatus* and 1868 *A. taeniorhynchus* on all three sites in Santa Cruz and Isabela for a total effort of 185 trap-nights. In the dry season of

2013 and with a total of 586 trap-nights, we captured a total of 840 *A. taeniorhynchus*, 300 *C. quinquefasciatus* and 3 *A. aegypti* on three sites in Santa Cruz and Isabela and two sites in the uninhabited island of Santiago. In the wet season of 2014 and following the same sites as in 2013, we captured a total of 6002 *A. taeniorhynchus*, 2130 *C. quinquefasciatus* and 7 *A. aegypti* with a total effort of 456 trap nights. In the dry season of 2015, we collected a total of 1011 mosquitoes consisting of 757 *A. taeniorhynchus* and 254 *C. quinquefasciatus* over 216 trap nights across 18 different sites on Isla Santa Cruz.

In 2012, we collected 1866 females and 2 males of *A. taeniorhynchus* and 2816 females and 158 males of *C. quinquefasciatus* on the two inhabited islands of Santa Cruz and Isabela. In 2013, we captured a total of 804 females and 36 males of *A. taeniorhynchus*, 289 females and 11 males of *C. quinquefasciatus* and 3 females of *A. aegypti* on Santa Cruz, Isabela and Santiago. In 2014 and following the same sites, we collected 5529 females and 473 males of *A. taeniorhynchus*, 1431 females and 700 males of *C. quinquefasciatus* and 7 females of *A. aegypti*. In 2015, we collected 38 males and 719 female *A. taeniorhynchus* and 26 males and 228 females of *C. quinquefasciatus*. For all sites, collections of mosquitoes was inversely proportional to elevation with mosquito abundances declining with increasing elevation and highest abundances of mosquitoes captured at low coastal elevations (Asigau et al. 2017).

Disease Screening

We screened a total of 13,692 abdomen and head/thorax regions of mosquitoes consisting of 8918 *A. taeniorhynchus*, 4764 *C. quinquefasciatus* and 10 *A. aegypti*. PCR screening for Plasmodium produced one positive result which belonged to a pool of 5 *A. taeniorhynchus* abdomens, collected from a light trap in Puerto Vilamil on Isabela in 2012. This gives a

prevalence of 0.03 percent (i.e.: number of positive infections divided by population size of female mosquitoes) for this site and season. Amplification of this sample resulted in a 100 percent match with Lineage A of the Plasmodium parasite, the same parasite infecting the endemic Galapagos penguin, *Spheniscus mendiculus* (Levin et al. 2009, 2013). This sequence also matched to the *A. taeniorhynchus* pool from Sorroco Island, Mexico (Carlson et al. 2011). We deposited this sequence into GenBank (accession number XXXX). We screened the corresponding head/thorax pool for positive individuals but amplification was unsuccessful. There are also no salivary gland smears from the PCR positive *A. taeniorhynchus* abdomen pool. We have reamplified the positive samples for *A. taeniorhynchus* consistently, diluted in 1:10 and have used this sample as an additional positive control along with the positive control found in *Spheniscus mendiculus*. Molecular screening also identified *Haemoproteus multipigmentatus*; *Haemoproteus* is one of the three genera besides *Plasmodium* and *Leucocytozoon* belonging to the order Haemosporida (Valkiūnas 2005) in abdomens of 5 individual *A. taeniorhynchus* captured in Puerto Ayora in 2015 via molecular screening. This gives a prevalence of 0.03 percent of infection in mosquitoes captured in the coastal elevation site of Puerto Ayora in 2015.

Microscopy

Salivary gland smears were prepared from 640 *C. quinquefasciatus* and 294 *A. taeniorhynchus* collected in the field season of 2012. Sporozoites of Plasmodium were not identified on any smears.

Discussion

Extensive sampling of mosquitoes across multiple seasons and years, multiple sites and multiple islands revealed their widespread distribution. Despite the widespread distribution of mosquitoes, our study revealed the low occurrence of avian malaria parasites in mosquitoes. We identified only one positive result of *Plasmodium* detected in a pool of *A. taeniorhynchus* abdomens. A lack of sporozoites from prepared salivary gland slides from PCR positive individuals does not provide evidence for disease competence in *A. taeniorhynchus*. To confirm vector competence, sporozoites should be present in salivary glands of field collected mosquitoes (Njabo et al. 2009). However, not all mosquitoes are capable of transmitting sporozoites by a bite, even when they are present in the salivary glands (Valkiunas et al. 2013). Gametocytes, which are the stage of the parasite found within red blood cells of avian hosts and infective to arthropod vectors, are taken up by feeding female mosquitoes. The gametocyte stage quickly progresses within the mosquito midgut to reproduce sexually and generate sporozoites which migrate to the insect's salivary glands, and await transmission to avian hosts. Given that *Plasmodium* was detected in abdomens of *A. taeniorhynchus*, we can infer that this arthropod species feeds or takes bloodmeals from birds infected with the *Plasmodium* Lineage A. This urges the need to understand the blood-feeding preferences and feeding host ranges of mosquitoes in the Galapagos.

Aedes taeniorhynchus is known to prefer feeding on mammals and reptiles over birds in Galapagos (Bataille et al. 2012). Animals known to be fed on by *A. taeniorhynchus* in Galapagos include marine iguanas, sea lions, Galapagos tortoises, bats, cattle, lava lizards, land tortoises, birds belonging to the Hirundinidae family, cormorants and even humans (Bataille et al. 2012; Asigau, pers. comm). Similarly, *C. quinquefasciatus* is an inherent opportunistic and generalist

feeder (Mboera and Takken 1999, Samuel et al. 2004) and feeds indiscriminately on birds and mammals (Zinser et al. 2004). In Galapagos, its diet has been found to be of a mammalian nature (Asigau, pers. comm). The high proportion of mammalian and reptilian bloodmeals by *A. taeniorhynchus* and *C. quinquefasciatus* in Galapagos could further lessen the chances of detecting avian malaria, which already occurs at low prevalence amongst endemic birds.

In Galapagos, the pool of mosquitoes found positive with the Lineage A Plasmodium parasite were captured in Puerto Vilamil on Isabela, an island site where Plasmodium parasites were previously detected (Levin et al. 2013). This suggests that this parasite could still be transmitted and maintained within the archipelago. However, the vertebrate hosts responsible for maintaining the transmission cycle of avian malaria remain unknown. This also urges the need to sample other vertebrate avian hosts such as introduced, migratory as well as sea birds in determining their role in the disease dynamics of avian malaria.

We also identified *Haemoproteus multipigmentatus* in the abdomen regions of *A. taeniorhynchus* collected in Puerto Ayora in Santa Cruz. *Haemoproteus multipigmentatus*, a parasite of the subgenus *Haemoproteus*, occurs at high intensities and prevalence in competent columbiform birds (Valkiūnas et al. 2010). The genus *Haemoproteus* has also been identified in swallow-tailed gulls (*Creagrus furcatus*), nazca boobies, red and blue footed boobies (*Sula sula* and *S. nebouxii*) and great and magnificent frigate birds (*Fregata minor* and *F. magnificens*) (Padilla et al. 2004, Levin et al. 2011). The presence of *Haemoproteus* in other passerines further indicates that there is evidence of parasitic spillover from doves to non-competent passerines (Jaramillo et al. 2017). *Haemoproteus* parasites are often non-pathogenic in adapted avian hosts (Bennett et al. 1993) but cause severe pathology in non-adapted birds (Olias et al. 2011) and can affect fitness in certain species (Valkiūnas 2005). Vectors known to transmit these parasites

include biting midges belonging to Culicoides (*Ceratopogonidae*) and hippoboscid flies (*Hippoboscidae*) (Valkiūnas 2005, Levin et al. 2012). However, experimental infection involving mosquitoes as vectors are needed to assess their role in the transmission of *Haemoproteus* amongst different avifauna species. Furthermore, the widespread distribution of mosquitoes in Galapagos where most of these *Haemoproteus* parasites were found suggests the need to investigate their role in disease transmission.

Conclusively, our study found a low prevalence of *Plasmodium* and *Haemoproteus* parasites in *A. taeniorhynchus* in the archipelago. Even though we did not find any of these parasites in *C. quinquefasciatus*, its association with avian malaria in Hawaii identifies it as a serious threat to avifauna in Galapagos. Distributional and seasonal patterns of both arthropod species reveal that they disperse from mainland Ecuador, between islands (Bataille et al. 2009a) and across different elevations (Bataille et al. 2010, Asigau et al. 2017). Since distributional patterns of mosquitoes are widespread, the conservation of endemic avifauna remains critical especially since conditions of mosquito and parasitic development coincide with avifauna ranges (Asigau and Parker 2018). It is of high importance that experimental studies understanding the vector competence of both species be established to manage avian malaria in the archipelago.

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Figures

Figure 1. Map of Galapagos with 100m elevation contour lines. Red dots show sampling sites and their elevations for; 1) Santa Cruz - Puerto Ayora (0m), Bellavista (180m) and Media Luna (500m); 2) Isabela - Puerto Villamil (0m), Zona Agricola (500) and Sierra Negra (878) and; 3) Santiago – Lagoon (0m) and Transition zone (180m). Note: Same map/sites used for Chapter One.

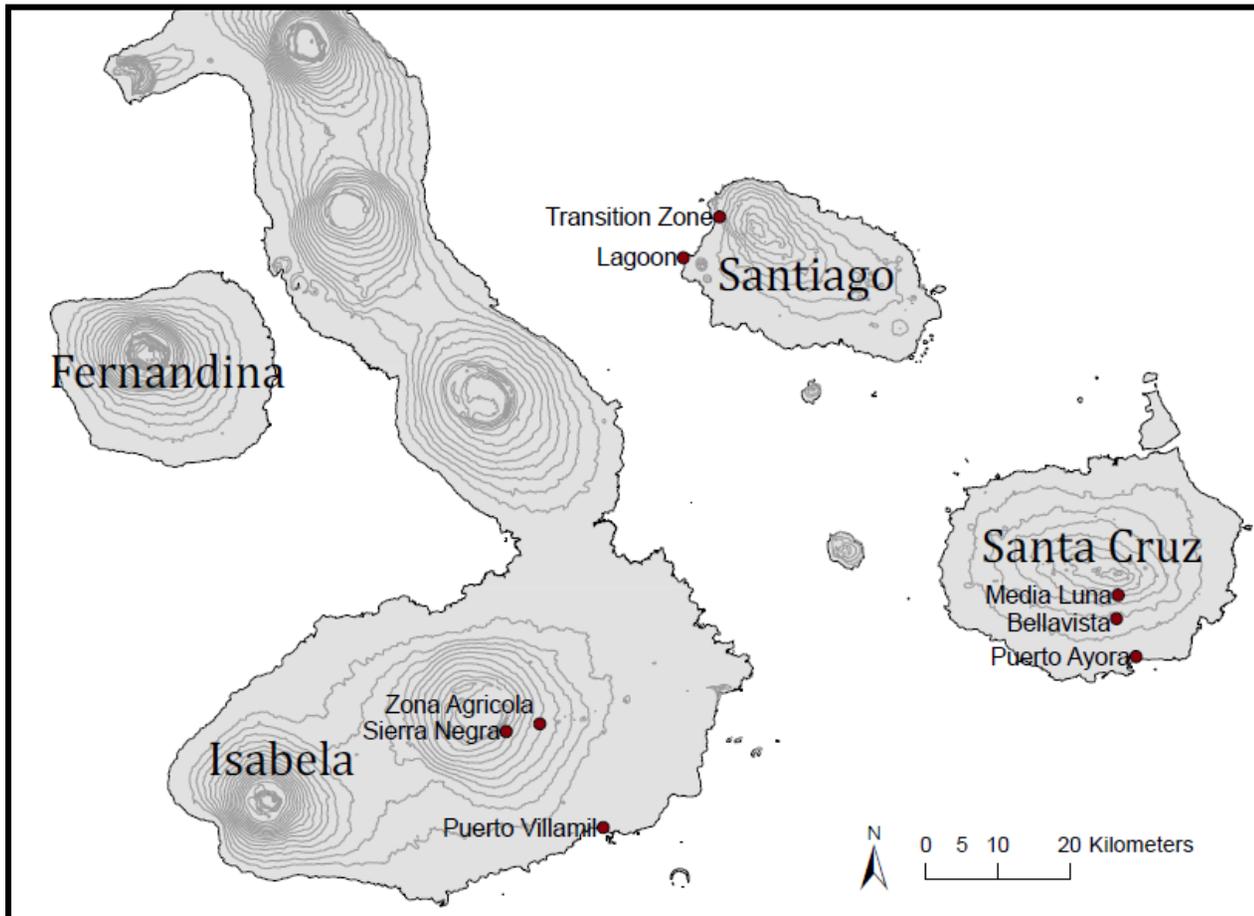
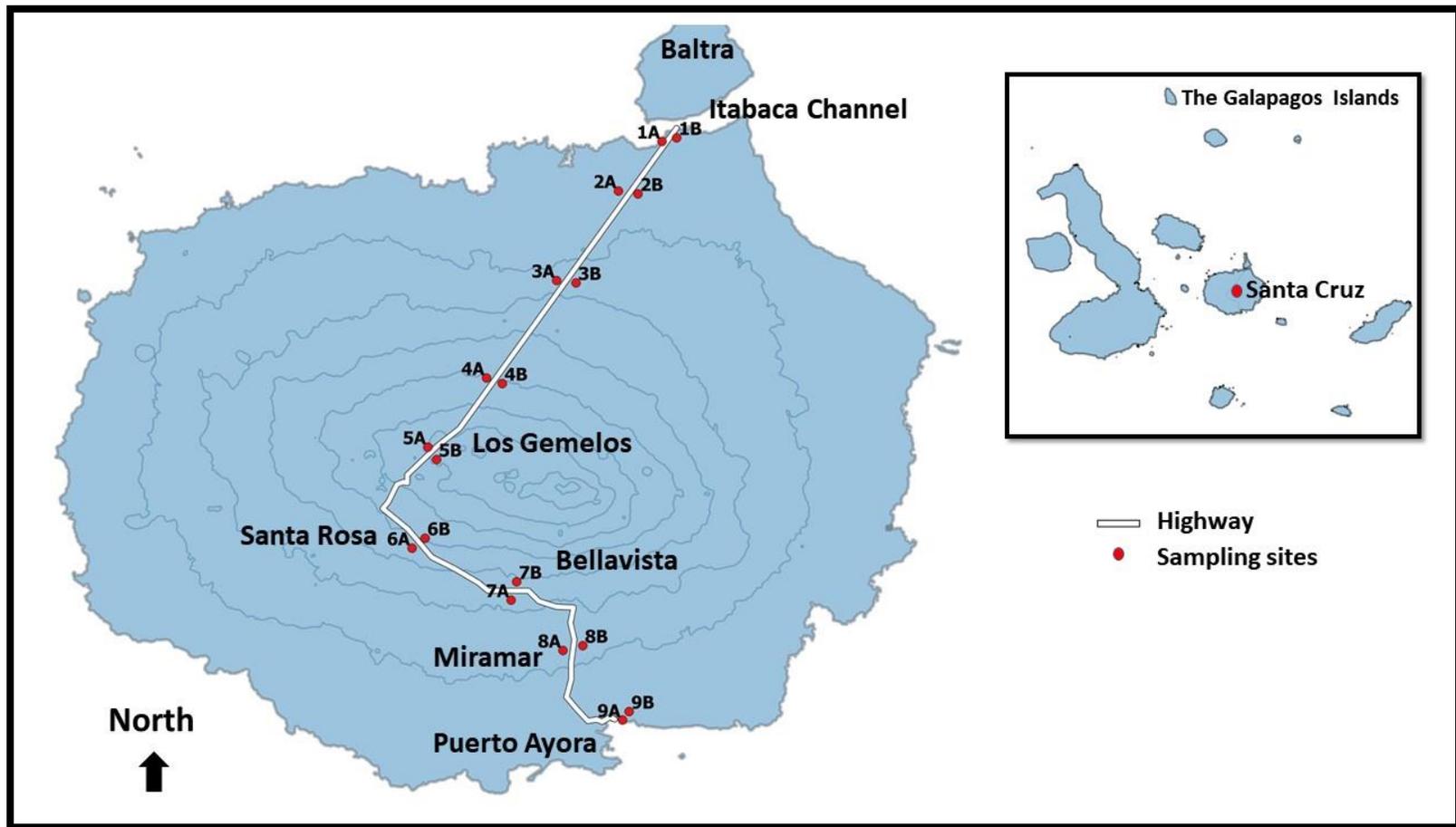


Figure 2: Map of 18 mosquito sampling sites extending from the most northern site, Itabaca Channel to the most southern site, Puerto Ayora. Names of localities (Itabaca Channel, Los Gemelos, Santa Rosa, Bellavista, Miramar and Puerto Ayora) are also indicated beside their corresponding mosquito sampling sites. Note: Same map/sites used for Chapter 2 and 3.



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