Title

Quantifying the roles of temperature and host community in the transmission of avian malaria in Hawai‘i

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QUANTIFYING THE ROLES OF TEMPERATURE AND HOST COMMUNITY IN THE TRANSMISSION OF AVIAN MALARIA IN HAWAIʻI

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by

Christa M. Seidl

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ABSTRACT

Quantifying the roles of temperature and host community in the transmission of avian malaria in Hawai‘i

Christa M. Seidl

Anthropogenic activities alter the host communities and environmental conditions that determine vector-borne pathogen transmission. Avian malaria (*Plasmodium relictum*) is a multi-host vector-borne parasite that infects numerous bird species. Avian malaria was recently introduced to Hawai‘i, where it is responsible for significant declines in native bird populations and is increasing in distribution with warming global temperatures. Our understanding of how anthropogenic changes to host communities and environmental conditions affect avian malaria transmission is limited because the roles of most host species are poorly understood, and the thermal responses of Hawaiian mosquito populations are undefined. My dissertation combines fieldwork and laboratory studies to quantify the infectiousness of different host parasite loads, or parasitemias, to mosquitoes, and it describes the influence of temperature on mosquito life history traits important for transmission. In my first chapter, I examined the effect of temperature on traits that determine population size for the primary vector in Hawai‘i, *Culex quinquefasciatus.* I described population-specific thermal response curves for larval development time, larval survival, and adult longevity. I used these data to predict adult mosquito

populations as a function of temperature across time and space. My results indicate *Cx. quinquefasciatus* populations in Hawai‘i have increased over the last decade and will continue to increase as global temperatures warm. In my second chapter, I quantify the relationship between two methods of parasitemia measurement: microscopy of blood smears and qPCR. I then examined the extent to which microscopy underestimates infection prevalence in Hawaiian bird species and found that underestimation differed among species due to differences in species' average parasitemias. In my third chapter, I quantified the effect of host parasitemia, day since mosquito feeding, and temperature on *Cx. quinquefasciatus* infectiousness, or the fraction of mosquitoes likely to transmit malaria. I found mosquito infectiousness increased gradually with increasing parasitemia, temperature, and the time since feeding. With this relationship, I estimated the infectiousness of parasitemias of wild-caught birds in Hawai‘i. The gradual shape of the relationship and considerable within-species variation in parasitemias led to many parasitemias, species, and communities creating infectious mosquitoes. As a result, most bird communities in Hawai‘i can sustain avian malaria transmission. This work provides a framework for determining the roles of host species and environmental conditions in avian malaria transmission and underscores the importance of using laboratory studies to quantify these relationships.

DEDICATION

I dedicate this dissertation to the ‘aina (land), the manu (birds), and the kanaka (people) of Hawai‘i.

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INTRODUCTION

One of the twenty-first century's top scientific priorities is understanding the transmission ecology of multi-host pathogens (Woolhouse et al. 2001). Multi-host pathogens are estimated to represent 60–75% of newly emerging diseases in humans (Cleaveland et al. 2001; Woolhouse 2002) and can pose significant threats to biodiversity conservation (van Riper et al. 1986; Frick et al. 2010; Cheng et al. 2011). The transmission of multi-host pathogens is determined by a community of host species that vary in their susceptibility, infectiousness, and contact rates (LoGiudice et al. 2003; Hamer et al. 2009; Johnson et al. 2013; Vanderwaal et al. 2014), and by environmental conditions, such as temperature and rainfall, which can influence host and pathogen biology (Harvell et al. 2002). Anthropogenic activities, including those causing species introductions and losses and climate change, alter both host community composition and environmental conditions (Dornelas et al. 2014), with complicated effects on pathogen transmission (LoGiudice et al. 2003; Kilpatrick et al. 2008; Rohr et al. 2011; Paull et al. 2017). Given this complexity, the transmission dynamics of multi-host pathogens within many ecological communities are poorly understood. This impedes accurate predictive modeling of transmission (Smith et al. 2009) and management of disease in affected populations (Leendertz et al. 2006; Wood & Lafferty 2013).

Avian malaria (*Plasmodium relictum*) is a multi-host wildlife disease for which an improved understanding of pathogen transmission is critical for biodiversity

conservation. Avian malaria (Phylum Apicomplexa: Order Haemosporidia) are vector-borne malaria parasites in the genus Plasmodium (Valkiūnas 2005). The generalist species, *P. relictum,* infects over 300 bird species, and host species vary substantially in their response to infection (Beadell et al. 2004; Fallon et al. 2005; Bensch et al. 2009; Svensson-Coelho et al. 2013; Dimitrov et al. 2015). In Hawai‘i, the introductions of *P. relictum* (lineage GRW4) (Warner 1968; Beadell et al. 2006), and its primary vector, the southern house mosquito (*Culex quinquefasciatus*) (Hardy 1960; van Riper et al. 1986; LaPointe et al. 2005), have contributed to the decline and extinction of multiple native Hawaiian bird species (Warner 1968; LaPointe et al. 2012). Several extant Hawaiian birds are highly susceptible to avian malaria and suffer from high parasitemias (fraction of red blood cells infected with parasites) and high mortality rates (van Riper et al. 1986; Atkinson et al. 1995, 2000; Yorinks & Atkinson 2000). The reduction of avian malaria transmission is a top priority for management efforts aimed at preventing further decline and extinction of the most endangered Hawaiian species (Haleakalā National Park 2022; Paxton et al. 2022).

Anthropogenic climate change and species introductions are affecting the transmission of avian malaria and may be exacerbating the risk of avian malaria-caused species declines. Many native birds are restricted to elevations where cold temperatures limit *Cx. quinquefasciatus* and *P. relictum* development (van Riper et al. 1986; LaPointe et al. 2005, 2010); however, precipitous population declines in the most susceptible native species have coincided with increased avian malaria prevalence at high elevations (Atkinson et al. 2014; Paxton et al. 2016). Warming

global temperatures and changing precipitation patterns are suspected drivers behind the increased abundance and elevational distribution of *Cx. quinquefasciatus* and *P. relictum* (Benning et al. 2002; Samuel et al. 2011; Paxton et al. 2016). Hawai‘i also contains over 50 non-native bird species introduced from North and South America, Africa, and Asia (Long 1981; Moulton & Pimm 1983; Pratt et al. 1987). Many of these species co-occur with native Hawaiian species and can be infected with avian malaria (van Riper et al. 1986; Atkinson et al. 2014; Neddermeyer et al. 2023). Non-native species are generally thought to be less susceptible and experience lower parasitemias and rates of mortality than native Hawaiian species (van Riper et al. 1986; Atkinson & Samuel 2010); however, the role of these species in avian malaria transmission is poorly understood. Transmission of avian malaria in Hawai‘i thus depends on the infectiousness of both native and non-native birds, their exposure to mosquitoes, and environmental factors, such as temperature, that influence host, vector, and pathogen interactions.

My dissertation research improves our understanding of transmission dynamics for avian malaria in Hawai‘i. My work addresses three broad questions: 1) How does temperature affect vector life history traits–particularly those associated with disease transmission? 2) How do the methods we use to detect host infection influence our understanding of pathogen transmission? 3) What is the relationship between host parasitemia and mosquito transmission, and what does the relationship mean for the role of different species in avian malaria transmission in Hawai‘i?

I addressed these questions through a combination of fieldwork and laboratory experimental infection studies that quantify some of the factors affecting avian malaria transmission in Hawai‘i. In my first chapter, I examined the effect of variable temperatures on the Hawaiian *Cx. quinquefasciatus* life history traits that influence pathogen transmission by determining mosquito population size. These are larval development time from egg hatch to adult emergence, larval survival to emergence, and adult longevity. I found larval development time and adult longevity of both sexes decreased with increasing temperature while larval survival to emergence increased. I used these data to calculate the number of "adult mosquito days", or the number of adult mosquitoes that would be produced from 100 eggs and how long they would live, as a function of temperature. I mapped this value across time, space, and elevation in Hawai‘i. The results indicate that adult mosquito days have increased in the last ten years and predict that with climate change, *Cx. quinquefasciatus* populations in Hawai‘i will continue to increase in size, including in habitats previously too cold for mosquito development.

My second chapter quantifies the relationship between two methods of parasitemia measurement and detection: microscopy analysis of blood smears, which was once the only technique to quantify *P. relictum* infection, and qPCR. I use this relationship to describe the extent to which microscopy underestimates infection prevalence in Hawaiian bird species. I estimate the likely infection patterns in birds for a large, important study done by microscopy. I found that the fraction of infections missed by microscopy differed substantially among birds due to differences

in species' parasitemia levels. Infection prevalence in non-native bird species with low average parasitemias was likely 4–5-fold higher than previous microscopy estimates. In contrast, adjusted prevalence was only 1.5–2.3-fold higher than original estimates for two native species with high average parasitemias. Thus, the prevalence of infection and therefore the potential contribution of many species to transmission was greatly underestimated by the previous study. Additionally, the relationship between the two methods also allowed me to convert the experimental infection studies that used microscopy to describe acute and chronic infections in Hawaiian species into qPCR values. I used this conversion to help answer key questions about the contribution of these stages to avian malaria transmission in my final chapter.

Finally, in my third chapter, I quantify the relationship between bird host parasitemia and mosquito infectiousness for *Cx. quinquefasciatus* mosquitoes across a range of *P. relictum* parasitemias, temperatures, and time since feeding. I used the relationship to estimate the infectiousness of 17 species of birds in Hawai‘i using 743 parasitemia estimates from wild-caught birds. I found that the relationship between mosquito infectiousness and bird parasitemia was gradual, not sharp, resulting in a wide range of parasitemias being partly infectious to mosquitoes, including parasitemias as low as 0.0004%. In addition, I found that parasitemia varied substantially within bird species. The combination of a gradual relationship and high within-species variability in parasitemia resulted in considerable overlap between the infectiousness of native and non-native birds. My results also allowed me to estimate the number of infectious mosquitoes created by the acute and chronic stage of avian

malaria infection in two species. I found that chronic infections create many more infectious mosquitoes and are therefore more important for avian malaria transmission than the high parasitemia acute stage for certain species.

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CHAPTER 1

The effect of temperature on life history traits of Hawaiian *Culex quinquefasciatus* mosquitoes

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Abstract

Climate change is altering the transmission of many vector-borne diseases. Characterizing the effect of temperature on the life history traits of specific vectors is important for understanding vector population dynamics and patterns of vector-borne disease transmission under current and future climates. Temperature influences vector population size, distribution, and pathogen competence, determinants of the rate and magnitude of pathogen transmission, by directly impacting many vector life history traits. In Hawai‘i, thermal performance curves for Hawaiian *Culex quinquefasciatus* Say (Diptera: Culicidae) life history traits are needed to improve control of avian malaria, *Plasmodium relictum*, a pathogen spread by *Cx. quinquefasciatus,* and which threatens many endemic bird species with extinction. Here, we quantify larval development time, larval survival to emergence, and adult longevity of male and female *Cx. quinquefasciatus* at five mean temperatures between 9.5 and 28.5˚C with ± 5 °C daily fluctuations. These regimes simulate average daily temperature profiles

for different elevations, seasons, and 2 °C of warming in Hawai‘i. We found larval development time and adult longevity of both sexes decreased with temperature while larval survival to emergence increased. Adults survived twice as long in the 13.5 °C temperature treatment than at 28.5 °C but needed four times longer to emerge. We integrated our results to calculate the number of "adult mosquito days", which are the number of adult mosquitoes that would be produced from 100 eggs and how long they would live, as a function of temperature. We projected these values onto maps of air temperature in Hawai‘i for the hottest and coldest months (August, January) in 2012, 2022, and 2 °C of warming to 2022. Across most of Hawai‘i, adult mosquito days increased slightly from 2012 to 2022 in both January (0.62%) and August (1.84%) and are predicted to increase much more with 2 °C of warming (January = 5.71.%; August = 3.48%), including in habitats above 1,500 meters elevation, where many disease-threatened bird species occur. Overall, our results suggest that *Cx. quinquefasciatus* populations in Hawai‘i will continue to increase in size, including in habitats previously too cold for mosquito development.

Introduction

Vector-borne diseases pose a significant threat to both human and wildlife health (Daszak et al., 2000; Kilpatrick & Randolph, 2012; Gould et al., 2017). Climate, including ambient temperature and precipitation, influences the spatial and temporal distribution as well as the intensity of vector-borne pathogen transmission by altering both vector and pathogen vital rates, vector competence, and host-vector

contact rates (Rueda et al., 1990; Purse et al., 2005; Kilpatrick et al., 2010; Paaijmans et al., 2013; Ciota et al., 2014; Kraemer et al., 2015). Over the past four decades, climate change has been associated with geographic expansion and increased incidence of many vector-borne diseases (Siraj et al., 2014; Gould et al., 2017; Watts et al., 2019). The relationships between temperature, vector life history traits (including larval development and survival and adult longevity), and pathogen transmission, vary across vector species and populations (Ciota et al., 2014; Sternberg & Thomas, 2014; Ruybal et al., 2016; Mordecai et al., 2019; Cator et al., 2020). However, most analyses of vector-borne disease transmission use the same thermal performance curves for geographically distant vector populations (Ahumada et al., 2004; Sternberg & Thomas, 2014; Ryan et al., 2019). This ignores the influence that local variation may have on a population's response to temperature, thereby reducing the accuracy of forecasts for a vector-borne pathogen's range and response to climate change.

Culex quinquefasciatus was the first mosquito species introduced to Hawai‘i, likely in 1826 (Hardy, 1960), and later introductions have increased its genetic diversity and structure among islands (Fonseca et al., 2000; Keyghobadi et al., 2006). This mosquito has contributed to the decline and extinction of native Hawaiian birds species by transmitting diseases such as avian pox (*Poxvirus avium*) and avian malaria (*Plasmodium relictum;* Warner, 1968; Van Riper et al., 1986; van Riper et al., 2002; LaPointe et al., 2005). As a result, many native birds are now restricted to elevations where cold temperatures limit *Cx. quinquefasciatus* and *P. relictum*

development (van Riper et al., 1986; LaPointe et al., 2005, 2010). However, climate change is shrinking these disease refugia (Benning et al., 2002; Paxton et al., 2016) by increasing the abundance and elevational distribution of *Cx. quinquefasciatus* and avian malaria (Benning et al., 2002; Samuel et al., 2011). The magnitude and timing of future changes to *Cx. quinquefasciatus* populations in Hawai‘i will depend on the relationships between temperature and the life history traits of local populations, but local data do not exist.

Our goal was to determine the effect of temperature on the life history traits of Hawaiian *Cx. quinquefasciatus,* and how these influence population dynamics and distribution. We measured three mosquito life history traits: larval development time from eggs hatch to adult emergence, larval survival to emergence, and adult longevity at five cycling temperature regimes (Figure S1) that simulated different elevations, seasons, and 2 °C of warming for both female and male mosquitoes from two wild-collected populations. We integrated our results to calculate the number the number of adult mosquitoes that would be produced from 100 eggs and how long they would live, as a function of temperature. We then mapped adult mosquito days values, the product of larval survival to emergence and adult longevity, onto the complex elevational topography of Hawai‘i using elevation-dependent temperatures. Our results demonstrate how temperature affects the life history traits of Hawai‘i-derived populations and contribute to our understanding of how climate change will impact mosquito-borne pathogen transmission in the Hawaiian Islands.

Materials and methods

Study populations

We collected *Culex quinquefasciatus* Say (Diptera: Culicidae) egg rafts from two locations on Hawai‘i Island: ten rafts from a site near the city of Hilo (elev. 22 m, Latitude 19.7035689, Longitude -155.0766949) on the windward side of the island in August 2019 and 6 rafts from a site near the town of Captain Cook on the leeward side of the island (elev. 205 m, 19.4613276, -155.8981797) in December 2019.

Rearing, handling, and temperature treatments

Egg rafts were hatched at 24 °C under a 12L:12D hrs. photoperiod. We divided first instar larvae between eight Tupperware plastic flats (Table S1; 276-424 larvae per flat for the Hilo population, 30 per flat for the Captain Cook population, except 1 flat of 50 at 28.5 °C). We filled flats with 1 liter of deionized water and fed larvae finely ground Kaytee Koi's Choice® Premium Fish Food daily. Two flats were placed into each of four environmental chambers programmed to simulate an average daily temperature profile for an elevation and time of year on Hawai‘i Island (Figure S1).

We used environmental chambers to create five temperature treatments (mean temperatures 9.5 °C, 13.5 °C, 19. 5 °C, 26.5 °C and 28.5 °C) with seven set points (Figure S1). These treatments simulated daily temperature profiles (range 5 °C) for months and elevations of interest using data from two National Oceanic and Atmospheric Administration (NOAA) stations in 2017-2019. The two warmest

treatments simulated the average hourly air temperatures for a lower elevation site (NOAA Climatological Data Station Hilo 5 S, elevation 189 m) during the average hottest annual month (August) with (mean 28.5 °C) and without (mean 26.5 °C) a 2 °C increase predicted by climate models (Benning et al., 2002; IPCC, 2007). The third treatment simulated the coldest month (January; mean 9.5 °C) at 1,950 m in Hakalau National Wildlife Refuge (NWR), a high-elevation forest established in 1985 for endangered forest birds. The two remaining treatments (means 13.5 °C, 19.5 °C) are intermediate between the hottest and coldest temperatures. We only had four environmental chambers and the populations were studied sequentially; the mosquitoes from Hilo were measured at 9.5 °C, 13.5 °C, 26.5 °C and 28.5 °C, and the Captain Cook mosquitoes were measured at 13.5 °C, 19. 5 °C, 26.5 °C and 28.5 °C (Figure S1).

We sexed and transferred emerging adults once daily in groups of 10 or fewer into 32 oz. collection cups. All adults were fed ad libitum on 10% sucrose-soaked cotton balls, and collection cups were checked daily to record adult mortality. Water dishes were kept inside each chamber to maintain relative humidity at 70-80% across all treatments.

Statistical methods

All statistical analyses were done in R 4.0.2 (R Core Team, 2022). We used a generalized linear model with a gamma distribution and an inverse link to assess the effects of average temperature, site, sex, and starting larval density on larval

development time (time in days from egg hatching until adult emergence). We used a similar model for adult longevity, using temperature, starting larval density, and sex as predictors of longevity, or number of days alive. We used logistic regression (a generalized linear model with a binomial distribution and a logit link) to assess the effects of temperature and starting larval density on larval survival to emergence, or the proportion of 1st instar larvae that emerged as adults. We examined both linear and quadratic temperature terms for development time and linear and quadratic larval density terms for larval survival to emergence because the residuals for the linear models displayed evidence of nonlinearity. Finally, we used larval development time, larval survival to emergence, and adult longevity values from our fitted models to populate a simple age-structured population model that calculated the daily change in abundance of 100 mosquitoes from each sex as they transitioned from larvae to adults, across a range of temperatures. Our simulation calculated the daily change in larvae and adult population abundance across time as individuals were lost to other life stages or temperature-dependent mortality and we assumed no immigration or reproduction. This was done for each temperature by first calculating larval daily survival (fraction of larvae that emerged^ (1/larval development time)), the adult morality rate (1/adult longevity) and adult daily survival (fraction of larvae that emerged^(1/adult longevity)). Mosquitoes then transition from larvae to a pupal stage (duration 1 day), and onto adults.

We used our fitted life history models to predict and map adult mosquito days across the state of Hawai‘i for past (2012), current (2022), and predicted future (2 °C

warmer than 2022) temperatures in the warmest (August) and coldest (January) months using 250 m resolution rasterized monthly mosaic average temperature maps downloaded from the Hawai‘i Climate Data Portal (HCDP; https://www.hawaii.edu/climate-data-portal/; Kodama et al., n.d.; J. McLean et al., 2021; J. H. McLean et al., 2020). These maps predict the average temperature (°C) for each pixel using piecewise linear regression with elevation as a predictor and data from 96 temperature stations across Hawai‘i (Kodama et al., n.d.). We predicted new grid values for adult mosquito days using a pixel's temperature and our fitted temperature models for adult emergence and survival. Adult mosquito day values are the product of the total number of emerging adults and their average adult longevity (days alive). We examined the changes in adult mosquito day values for three years (2012, 2022, and 2022 + 2 °C), two seasons (winter = January, summer = August), and above 1,500 m above sea level, where many disease-threatened bird species occur (Camp et al., 2009).

Results

Life history traits

A total of 1,290 mosquitoes emerged from egg rafts, 1,105 from the Hilo site and 185 from the Captain Cook site. Across all temperatures, 464 females and 641 males from Hilo and 97 females and 88 males from Captain Cook emerged as adults. All larvae in the coldest treatment (mean 9.5 °C) died by day 37 post-hatch after reaching second and third instar stages. Temperature, larval density, and sex affected

development time, with the effect of temperature differing between sites (Figure 1B; Table S2). Between 13.5 °C and 28.5 °C, development time decreased nonlinearly with increasing temperature from 40.43 (SD ± 5.32) d at 13.5 °C to 9.50 (SD ± 1.17) d at 28.5 °C. Males emerged approximately 1 day sooner across temperatures than females and larval development time decreased with increasing larvae densities (Table S2). Mosquitoes from Hilo emerged on average 5–25% faster than mosquitoes from Captain Cook from the same temperature treatments. The fraction of larvae that emerged as adults increased with increasing temperature, and decreased with increasing larval density (Figure 1A, Table S3). The fraction of larvae emerging as adults approximately doubled from 37% (SD ± 0.11) at 13.5 °C to 83% (SD ± 0.20) at 28.5 °C. The longevity of adult mosquitoes decreased with increasing temperature and decreased with increased larval density (Table S4; Figure 2). Longevity for both females and males was almost twice as long at 13.5 °C as at 28.5 °C (Figure 2).

We integrated the models for the traits above to simulate the number of larvae and adult mosquitoes (male and female) surviving over time from 100 eggs from the Hilo population reared at a larval density of 320 mosquitoes per L (Figure 3). Warmer temperatures produced more adults, which emerged earlier and maintained higher populations for longer than cooler temperatures. The warmest 28.5 °C temperature produced the highest number of emerging adults due to higher larval survival, with the peak in adults occurring 12-13 days post-hatch, and it maintained the highest population of adults over time (Figure 3). At day 100, the adult female population was 1.5 times greater at 28.5 °C than at a temperature 2 °C cooler and 7.2 times

greater than at a temperature 9 °C cooler. The same pattern was observed for male populations in these temperatures.

Past, present, and future adult mosquito days mapping

Adult mosquito days increased with increasing temperature between 13.5 °C and 28.5 °C (Figure 4). There was an increase in adult mosquito days across Hawai‘i between 2012 and 2022 in January (0.62%) and August (1.84%). With an additional 2 °C of warming, adult mosquito days are predicted to increase much further (January = 5.71%, August = 3.48%), because adult mosquito days increase with temperature across the range of temperatures observed in Hawai‘i (13.5 °C to 28.7 °C; Figure S2). In areas above 1,500 m asl where temperatures were at or above 13.5 °C, the minimum temperature in which we observed larval development to emergence, adult mosquito days slightly decreased in January (-0.17%) but increased in August (2.49%) between 2012 and 2022. With an additional 2 °C of warming, adult mosquito days are predicted to increase much more in both January (6.94%) and August (6.24%).

Discussion

Climate change models predict many tropical regions, including the Hawaiian Islands, will experience an average temperature increase of 2 °C by the end of the 21st century (Still et al., 1999; Benning et al., 2002; IPCC, 2007). Such a rise is likely to impact the biology, distribution, and vector competence of *Cx. quinquefasciatus*

mosquitoes (Richards et al., 2010; Ciota et al., 2014; Samy et al., 2016; Paull et al., 2017; Ryan et al., 2019). Consistent with other studies, our results demonstrate that temperature has strong effects on *Cx. quinquefasciatus* larval development, larval survival, and adult longevity (Lachmajer & Hien, 1975; Rueda et al., 1990; Ahumada et al., 2004; Ciota et al., 2014; Mordecai et al., 2013). These life history traits are commonly used to predict how climate shifts alter mosquito-borne disease transmission (Dye, 1992; Rogers & Randolph, 2000, 2006). We examined the responses of both sexes of Hawaiian *Cx. quinquefasciatus* to temperatures that they are likely to experience seasonally across their distributions in Hawai‘i (Benning et al., 2002), and our results suggest that warming will lead to an expansion in adult mosquito days, indicating an increase in *Cx. quinquefasciatus* adult mosquito populations in many locations.

We observed non-linear relationships between temperature and both adult and larval life history responses, and our results reflect the considerable variability in these responses found in other studies. Larval development time in our study decreased sharply with temperature, with a non-linear relationship that was very similar to other studies of *Cx. quinquefasciatus* (Figure S3; Rueda et al., 1990; Mordecai et al., 2013; Ciota et al., 2014; Moser et al., 2023). In contrast, the relationship between fraction of larvae surviving to emerge and temperature was highly variable across studies. We found an overall increase in larval survival with temperature as did a study of a lab colony from Japan (Oda et al., 1999), and a study of field-collected larvae from Argentina (Grech et al., 2015). However, in three other

studies that included both laboratory colonies and field-collected larvae from multiple locations, one found a unimodal relationship with a peak at 25 °C and a sharp drop at 34 °C (Rueda et al., 1990), one found a linear decrease from 20 °C to 32 °C (Ciota et al., 2014), and the last found little variation with temperature (Figure S3; Ciota et al., 2014). Studies of other *Culex* and *Aedes* mosquito species have often found unimodal relationships with species-specific or population-specific optima, but only when both high and low temperatures were examined (Ruybal et al., 2016). In adults, we found longevity of both sexes decreased non-linearly with temperature, in agreement with most other studies (Figure S3; Moser et al., 2023). However, the values and shapes of longevity-temperature relationships can vary considerably (Figure S3). We observed a sharp decline between 13.5 °C and 19.5 °C but little further decline above 19.5 °C, whereas another study found strong declines above 20 °C in both field and laboratory colonized mosquitoes (Ciota et al., 2014); a third study used a narrow temperature range and found no clear pattern (Oda et al., 1999). Studies of multiple populations of the same mosquito species might shed light on the factors influencing the shape of relationships between temperature and life history traits, but a relatively large number of populations may be needed to disentangle the multiple potential causes.

The minimum temperature for *Cx. quinquefasciatus* larval development is not precisely known, despite its importance for delimiting the distribution of self-sustaining populations. Prior to this study, the lowest documented temperature to produce adult *Cx. quinquefasciatus* was 15 °C, with adult emergence after 31 days (Rueda et al., 1990). We found adults emerged at 13.5 °C after 40 days, but all larvae

failed to develop at 9.5 °C and died. This suggests that mosquitoes will not be able to complete development in January at elevations of 1,950 m or higher where temperatures are below 9.5 °C. However, lack of suitable conditions for larval development does not preclude adult mosquito presence at locations with average temperatures at or below 9.5 °C. Adults can survive at temperatures lower than those required for ontogeny (Tekle, 1960; LaPointe et al., 2010) and wind-assisted dispersal is well-documented in mosquitoes (Bailey et al., 1965; Service, 1997; Verdonschot & Besse-Lototskaya, 2014). Strong trade winds, El Nino storms, and hurricanes can lead to upslope dispersal by mosquitoes in Hawai‘i (Schroeder, 1993). Mosquito dispersal could also result in female *Cx. quinquefasciatus* reaching high-elevation habitats; mean dispersal distances were 1-2 km in Hawaiian rainforests (Lapointe, 2008) and other habitats (Fussell, 1964; Reisen et al., 1991; Lapointe, 2008; Medeiros et al., 2013). Additionally, with 2 °C of warming, locations that currently have average January temperatures of 9.5 °C will have temperatures that exceed 13.5 °C for 5 months of the year (June-October; Giambelluca et al., 2014), allowing adult mosquitoes to develop locally during these months. Thus, mosquito dispersal from lower elevations and future warming could lead to adults being present in areas and in seasons where they are not currently capable of developing (Freed & Cann, 2013). Dispersing adult mosquitoes and mosquitoes developing locally in warmer months may contribute to sporadic avian malaria and pox outbreaks in high-elevation native bird communities (Freed & Cann, 2013; Samuel et al., 2018).

Our results suggest that warmer temperatures will produce larger and more

persistent mosquito populations throughout Hawai‘i. Adult mosquito days were highest at the warmest temperatures because of substantially higher larval survival to emergence and only slightly lower adult longevity at 28.5 °C compared to 19.5 °C (Figures 1,2,3). If wild populations show similar trends in longevity and larval survival to emergence with temperature as we found in the lab, recent and future warming may increase mosquito populations in both low elevations and above 1,500 m asl in Hawai‘i (Figure 4). Whether populations of mosquitoes at high elevations will sustain transmission of avian malaria will depend on pathogen development rates, mosquito host preferences, and the parasitemias of hosts that are fed. Predicting future patterns of avian malaria transmission in Hawai‘i will require laboratory experiments, field studies, and mathematical modeling of host-vector-parasite interactions.

Conclusion

Understanding variation in thermal performance curves among vector populations could contribute to more accurate predictions of how climate change will affect vector-borne disease transmission. We conducted laboratory experiments to examine how current and future temperatures affected life history traits of two populations of *Cx. quinquefasciatus* from Hawai‘i in laboratory experiments. We found that Hawaiian populations of *Cx. quinquefasciatus* had similar responses to temperature for larval development time as studies of other populations but differed in patterns of larval and adult survival with temperature. Specifically, we found an

increase in larval survival with temperature and a relatively weak trend in adult longevity with temperatures above 19.5 °C. The combination of these two patterns suggests that future warming will increase populations of *Cx. quinquefasciatus* in Hawai‘i and likely lead to an upslope expansion in self-sustaining mosquito populations, with the potential to further spread disease to endemic bird species already under threat (Paxton et al., 2016).

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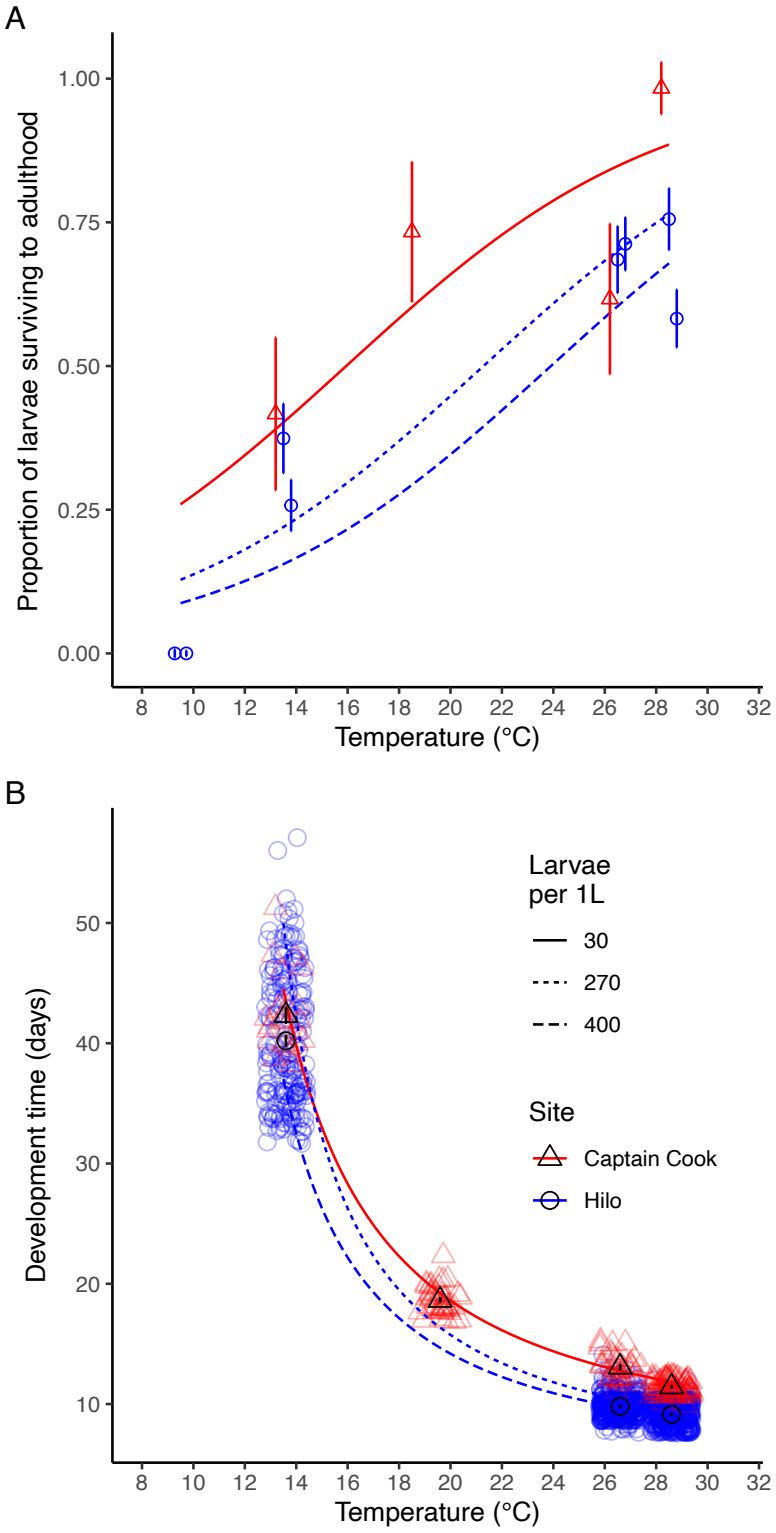
Figures

Figure 1. *Culex quinquefasciatus* larval survival and development time plotted against temperature. **A)** Points show average fraction of larvae surviving and emerging as adults ± SE plotted against temperature. Color indicates the starting larval number per L and population (Site). Lines show fitted model estimates (Table S3). All larvae died at 9.5 °C. **B)** Points colored by population (Site), show values for individual mosquitoes, and are jittered across the x-axis to facilitate presentation. Black shapes show mean development time ± SE. Lines show the fitted model (Table S2) for development time in days from hatching to adult emergence by site and for different numbers of starting larvae per 1L.


A
Proportion of larvae surviving to adulthood
1.00
0.75
0.50
0.25
0.00
8
10
12
14
16
18
20
22
24
26
28
30
32
Temperature (°C)
B
Development time (days)
50
40
30
20
10
Larvae
per 1L
30
270
400
Site
Captain Cook
Hilo
Temperature (°C)

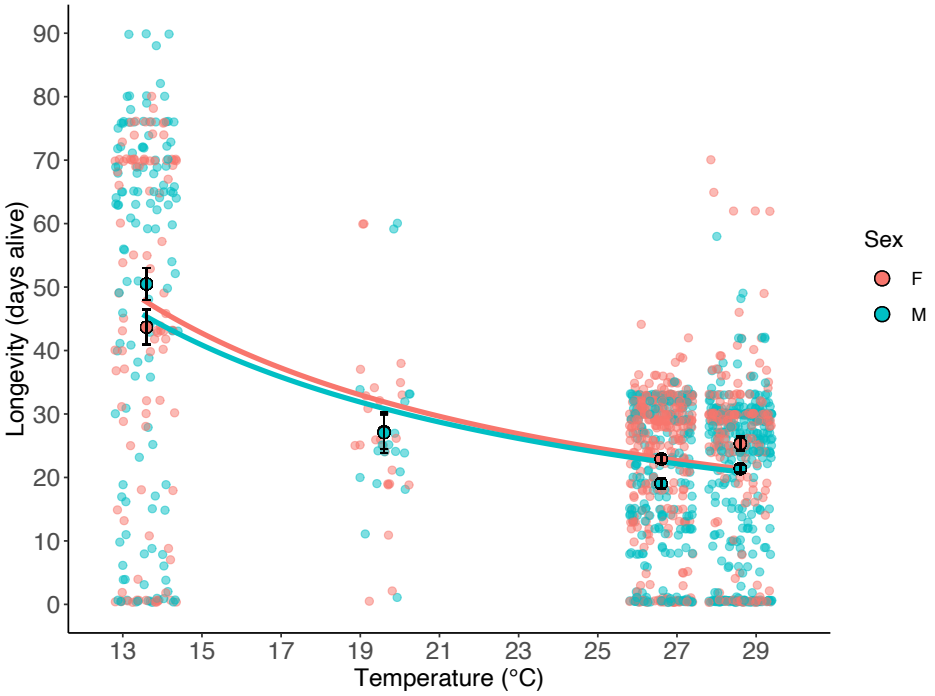


Figure 2. *Culex quinquefasciatus* adult longevity plotted against temperature. Points show values for individual mosquitoes and color indicts sex: red = female (F) and blue = male (M). Points with black outlines show mean longevity ± SE for each sex within a temperature. Points are jittered in the x-direction to facilitate presentation.

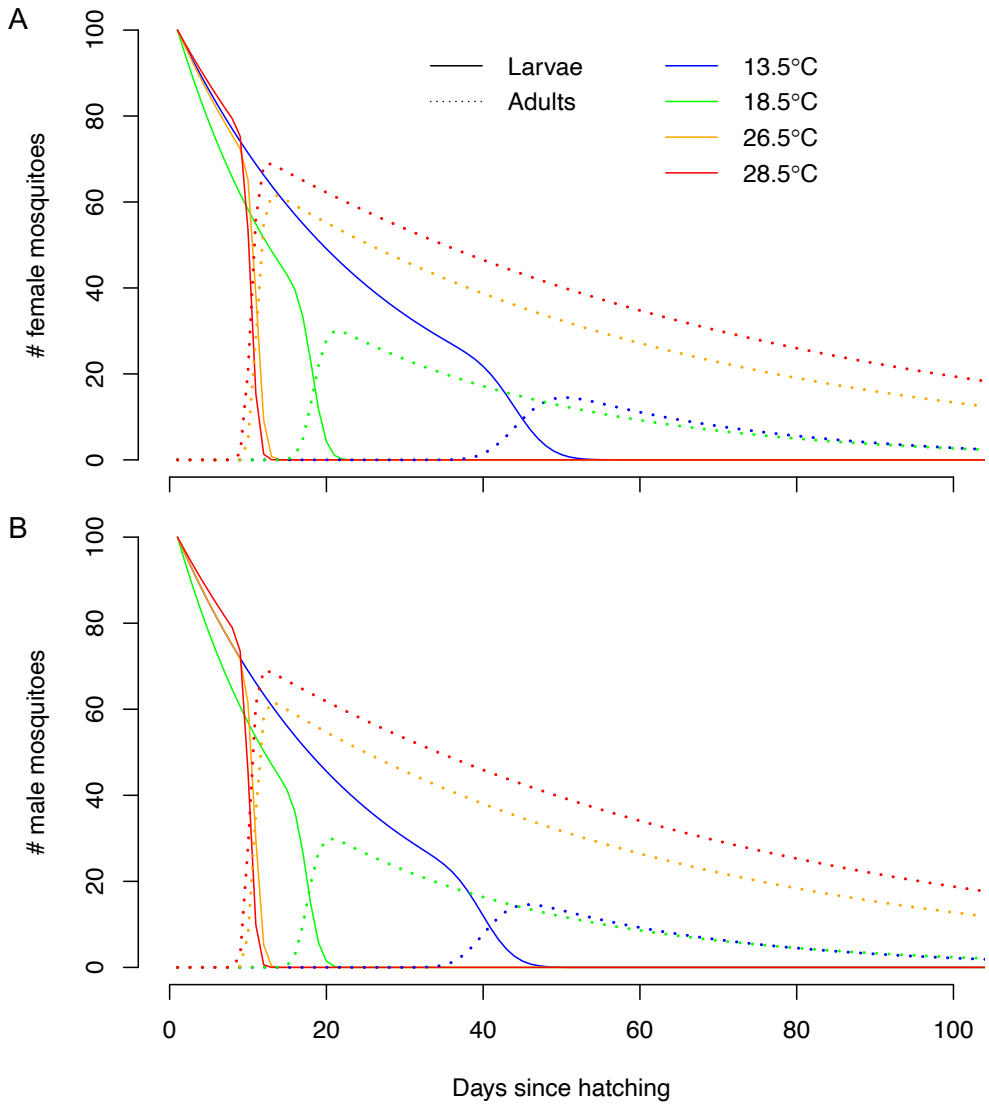


Figure 3. Simulated population dynamics for 100 **A)** female and **B)** male *Cx. quinquefasciatus* mosquitoes from the Hilo population. Each line color shows the fate of 100 larval mosquitoes over time, starting at hatch day 0, for a specific temperature and a larval density of 320 larvae per L.

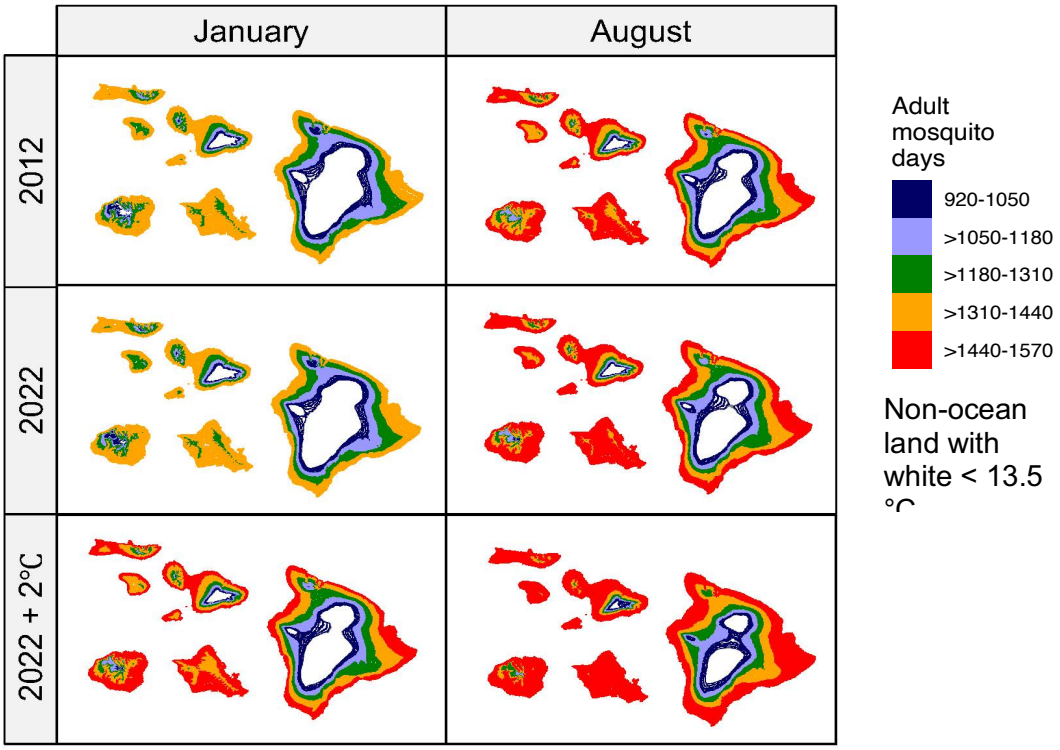


Figure 4. Number of adult mosquito days for *Culex quinquefasciatus* mapped across the Hawaiian islands using temperatures from the coldest (January) and warmest (August) months of the year using past (2012), current (2022), and predicted future temperatures (2022 + 2 °C). Adult mosquito days are the average number of 100 larvae that survive and emerge as adults*average adult longevity using the average temperature for a month and year. Adult mosquito days are not calculated for temperatures below 13.5 °C, the lowest average temperature in which we observed development to emergence

Supplemental materials

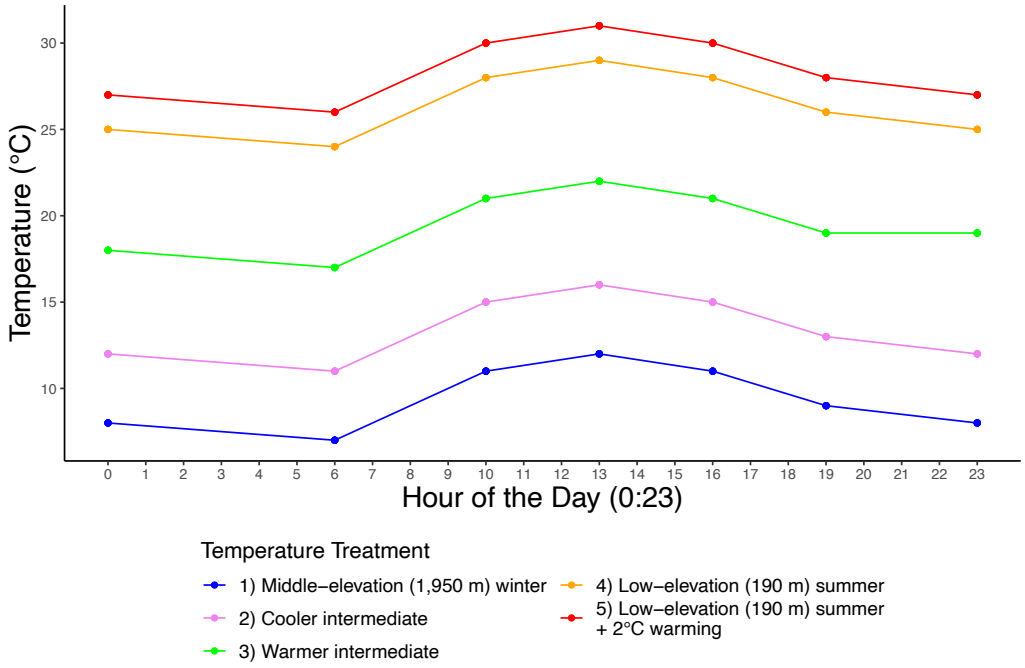


Figure S1. Daily temperature set points for each temperature treatment. Each treatment simulates average hourly and daily temperatures for an elevation and month of interest on the Island of Hawai‘i from 2017–2019 (range 5 °C).

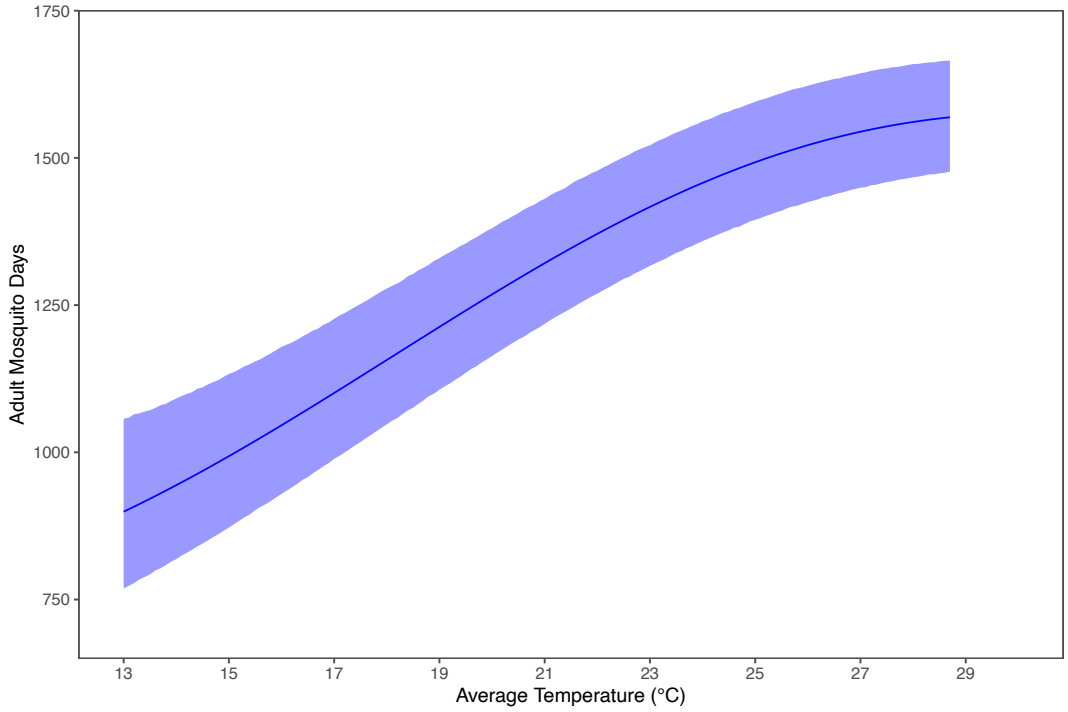


Figure S2. Adult mosquito days plotted against temperature. We estimated adult mosquito days by multiplying the average number of larvae, out of 100, that would survive to emerge, and average adult longevity at each temperature using the fitted models (Figure 1). Line shows predicted relationships with 95% confidence intervals.

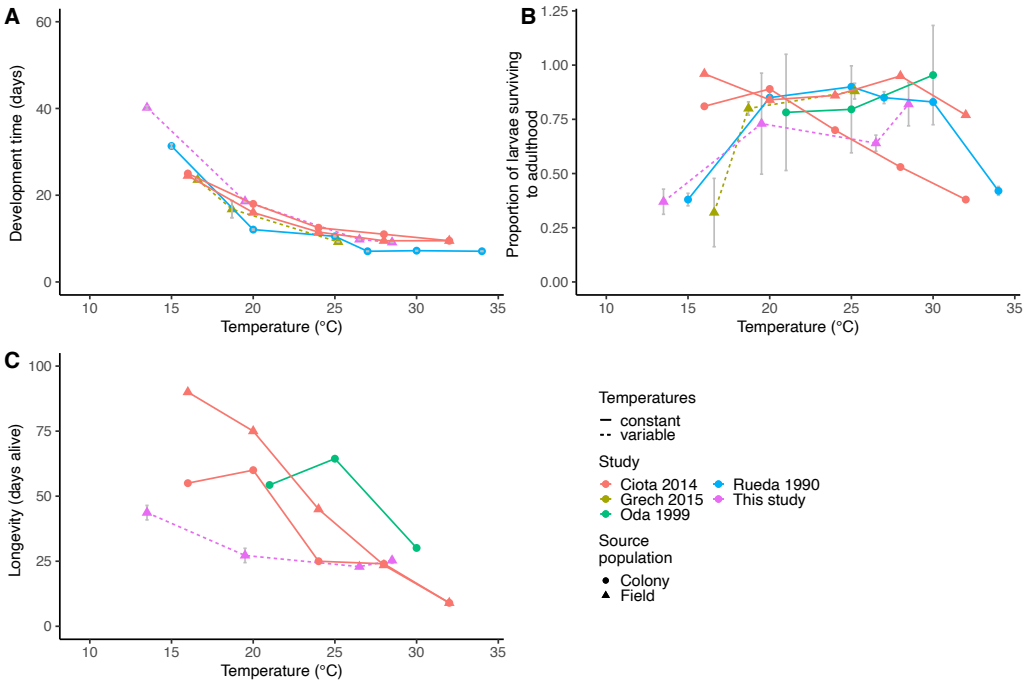


Figure S3. A comparison of temperature and **A)** larval development time, **B)** larval survival to emergence, and **C)** longevity relationships from available data in five *Culex quinquefasciatus* life history studies. Points are averages from each study ± SE bars. Wild-collected mosquitoes were obtained from Orange County, CA, USA (Ciota et al., 2014), Córdoba City, Córdoba Province, Argentina (Grech et al., 2015), and Hilo, Hawai‘i, HI, USA (This study); laboratory colonies were originally obtained from wild mosquitoes collected in North Carolina, USA (Rueda et al., 1990) and Okinawa, Japan (Oda et al., 1999).

Table S1. Number of starting *Culex quinquefasciatus* mosquito larvae in each 1L flat of water from two populations held at each average temperature (T_{Ave}). Data reflect number of adults that emerged from each flat, larval survival, and sex ratio of adults emerging.

Population	T_{Ave}	flat #	# larvae	# adults	Larval survival	% adults female
Hilo	28.5	1	267	204	0.76	19.6
		2	410	233	0.57	29.6
	26.5	3	276	185	0.67	58.4
		4	424	285	0.67	58.6
	13.5	5	278	101	0.36	42.6
		6	374	103	0.28	31.1
	9.5	7	268	0	0.0	0
		8	388	0	0.0	0
Captain Cook	28.5	9	30	29	0.97	65.5
		10	50	50	1.0	44.0
	26.5	11	30	16	0.53	25.0
		12	30	21	0.70	47.6
	19.5	13	30	15	0.50	66.7
		14	30	29	0.97	51.7
	13.5	15	30	16	0.53	62.5
		16	30	9	0.30	77.8

Table S2. Results from a generalized linear model with a gamma distribution and an inverse link for larval *Culex quinquefasciatus* development time (days from egg hatch to adult emergence). A data point was an individual mosquito.

Predictor	Coefficients	SE	t value	Pr(>|t|)
Intercept	0.0685	0.000469	145.9	< 0.001
Temperature	0.8088	0.00977	82.8	< 0.001
Temperature^2	-0.0440	0.007031	-6.25	< 0.001
Site(Hilo)	0.00162	0.000858	1.89	0.0589
Larval starting density	0.000054	0.000002	23.8	< 0.001
Sex(Male)	0.00239	0.000225	10.6	< 0.001
Temperature*Site(Hilo)	0.285	0.01096	26.0	< 0.001
Temperature^2*Site(Hilo)	-0.0267	0.0126	-2.12	0.0338

Table S3. Results from a generalized linear model with a binomial distribution with a logit link of the fraction of *Culex quinquefasciatus* larvae surviving to adulthood. A data point was an individual mosquito.

Predictor	Coefficients	SE	t value	Pr(>|t|)
Intercept	-3.12	0.154	-20.3	< 0.001
Temperature	0.162	0.00628	25.8	< 0.001
Larval starting density	-2.33	0.3025	-7.71	< 0.001
Larval starting density^2	0.08030	0.159	0.505	0.614

Table S4. Results from a generalized linear model with a gamma distribution and inverse link of adult *Culex quinquefasciatus* longevity. Note that the inverse link means that the direction of an effect is the opposite of the sign (e.g., increasing temperature reduces longevity). A data point was an individual mosquito.

Predictor	Coefficients	SE	t value	Pr(>|t|)
Intercept	-0.00384	0.00213	-1.807	0.0710
Temperature	0.00172	0.0000824	20.9	< 0.001
Larval starting density	0.000454	0.000444	1.023	0.3070
Sex(Male)	0.001071	0.00113	0.951	0.342

CHAPTER 2

Linking avian malaria parasitemia estimates from quantitative PCR and microscopy reveals new infection patterns in Hawai‘i

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Abstract

Plasmodium parasites infect thousands of species and provide an exceptional system for studying host-pathogen dynamics, especially for multi-host pathogens. However, understanding these interactions requires an accurate assay of infection. Assessing *Plasmodium* infections using microscopy on blood smears often misses infections with low parasitemias (the fractions of cells infected), and biases in malaria prevalence estimates will differ among hosts that differ in mean parasitemias. We examined *Plasmodium relictum* infection and parasitemia using both microscopy of blood smears and quantitative polymerase chain reaction (qPCR) on 299 samples

from multiple bird species in Hawai‘i and fit models to predict parasitemias from qPCR cycle threshold (Ct) scores. We used these models to quantify the extent to which microscopy underestimated infection prevalence and to more accurately estimate infection patterns for each species for a large historical study done by microscopy. We found that most qPCR-positive wild-caught birds in Hawai‘i had low parasitemias (Ct scores ≥ 35), which were rarely detected by microscopy. The fraction of infections missed by microscopy differed substantially among eight species due to differences in species' parasitemia levels. Infection prevalence was likely 4–5-fold higher than previous microscopy estimates for three introduced species, including *Zosterops japonicus*, Hawai‘i's most abundant forest bird, which had low average parasitemias. In contrast, prevalence was likely only 1.5–2.3-fold higher than previous estimates for *Himatione sanguinea* and *Chlorodrepanis virens*, two native species with high average parasitemias. Our results indicate that relative patterns of infection among species differ substantially from those observed in previous microscopy studies, and that differences depend on variation in parasitemias among species. Although microscopy of blood smears is useful for estimating the frequency of different *Plasmodium* stages and host attributes, more sensitive quantitative methods, including qPCR, are needed to accurately estimate and compare infection prevalence among host species.

Introduction

Parasites are frequently used to test ecological and evolutionary hypotheses of coevolution, sexual selection, host immunocompetence, and pathogen virulence (Hamilton, 1982; Woolhouse et al., 1997; Otto and Nuismer, 2004; Mayer et al., 2016). Avian haemosporidian blood parasites are a common model system for these studies because they often infect multiple host species (Ricklefs and Fallon, 2002; Beadell et al., 2006; Fecchio et al., 2021), and hosts vary in their responses to infection (Atkinson and van Riper, 1991; Valkiūnas, 2005). Some hosts experience high parasite intensities and mortality when parasitized, while others suffer little morbidity and mortality, and many species maintain chronic low intensity infections for a number of years (van Riper et al., 1986; Valkiūnas, 2005). Variation in infections and host responses to infections influence transmission, evolution, and disease risk within host communities (Woolhouse et al., 1990, 1997; Regoes et al., 2000). A crucial part of studying host-parasite interactions is accurately measuring a host's infection status and the infection intensity.

Measuring infection prevalence and intensity is important in determining which host species are more likely to become infected (Beadell et al., 2006; Maria et al., 2009; Svensson-Coelho et al., 2013) and, for vector-borne diseases, estimating host infectiousness for vectors (Komar et al., 2003; Mackinnon and Read, 2004; Kilpatrick et al., 2007; Pigeault et al., 2015). The probability of detecting infection increases when infection intensity is higher, and infection intensity varies both among hosts and over the course of infection (van Riper et al., 1986; Moens et al., 2016).

The parasitemia (the fraction of red blood cells infected) for most malaria parasites is highest during the acute phase of infection, which lasts for a few weeks starting approximately one week after infection (Atkinson et al., 2000, 2001; Dimitrov et al., 2015), and hosts may suffer morbidity or mortality during this time and thereafter. Subsequently, species and individuals will remain chronically infected, often at much lower infection intensities, for months or years (Jarvi et al., 2002; Valkiūnas, 2005). Accurately estimating infection prevalence in a species or population can be challenging because heavily infected hosts can have decreased activity (Yorinks and Atkinson, 2000), which may lead to undersampling, especially in studies that capture flying birds with mist nets (Valkiūnas, 2005; Mukhin et al., 2016), and low-level chronic infections can be difficult to document, depending on the detection technique used (Jarvi et al., 2002).

Parasite detection methods vary in their sensitivity (probability of detecting true positives) and in the information they provide. Malaria researchers have employed different detection methods over time, with earlier studies using microscopy of stained blood smears and more recent studies primarily using PCR-based molecular techniques (Garnham, 1966; Fallon et al., 2003; Zehtindjiev et al., 2008). Microscopy provides useful information about the morphology and life stages of haemosporidians (Jarvi et al., 2002; Fallon et al., 2003; Valkiūnas et al., 2008), but it generally has lower sensitivity than molecular techniques for detecting infection. This is especially true for low infection intensities, because the number of red blood cells that can be reasonably screened are far less than the numbers in an extracted

DNA sample (Jarvi et al., 2002; Fallon et al., 2003; Fallon and Ricklefs, 2008). As a result, many studies now use either PCR (Beadell and Fleischer, 2005) or quantitative PCR (qPCR) to measure infection prevalence and the latter to estimate infection intensity (Neddermeyer et al., 2023; Paxton et al., 2023). However, integrating historical estimates of prevalence and intensity based on microscopy with more recent disease surveys that use qPCR requires a way to accurately integrate the measurements from these two methods.

Here, we develop a statistical relationship between *Plasmodium relictum* parasitemia quantified by microscopy and by qPCR to integrate past and current studies of avian malaria and examine patterns of infection among species. We analyzed blood samples both from experimentally infected birds and wild-caught birds in Hawai‘i (USA) and examined the probability of detecting infection in eight common species found in Hawai‘i that differ in parasitemia. We re-examined historical patterns of infection prevalence among these species using a large previous study that assessed infection using microscopy (van Riper et al., 1986). Finally, we combined parasitemia data from experimental infections and wild caught birds to assess whether parasitemias estimated from birds caught with mist nets are biased towards low results because birds with high parasitemias are less likely to be caught and sampled.

Materials and methods

Sample collection

We collected 720 avian blood samples by brachial venipuncture (25–100 µL) from wild birds caught in forested sites between 29–2,000 meters above sea level from 15 species on the islands of Kaua‘i, Oahu, Maui, and Hawai‘i (Hawai‘i, USA) between 2015-2022. We also took blood samples from Hawai‘i ‘amakihi (*Chlorodrepanis virens*), and domestic canaries (*Serinus canaria*) in the first 45 days after experimental infection with *P. relictum*. Canaries were infected between 2020 and 2022 by intramuscular injection with lineage GRW4 of *P. relictum,* using infected whole blood from wild birds caught on Hawai‘i Island or *P. relictum* passaged one to four times in canaries. *Chlorodrepanis virens*, which were captured from Upper Waiākea Forest Reserve (19° 38′N, 155° 21′W, 1,635 m), were infected by mosquito bite or intramuscular injection using a thawed, deglycerolized, aliquot of *P. relictum* GRW4 (KV115) passaged four times in canaries in 2015 (Paxton et al., 2023). Thin smears were prepared on glass slides from a drop of whole blood for some wild-caught bird samples and all laboratory-infected bird samples. Slides were air dried for 30 min., fixed for 1 min in 100% methanol (Spectrum Chemical Mfg. Corp, New Brunswick, NJ, USA) and stained for 1 h, within 24 h of preparation, with a 10% working solution of Giemsa stain stock in buffered solution (Ricca Chemical Company, Arlington, TX, USA). The remaining blood was placed immediately in ~1 mL of prepared Queen's Lysis Buffer (Seutin et al., 1991) and stored at room temperature for 1–90 days or frozen at -20 °C before qPCR analysis. All fieldwork

was conducted under Hawai‘i Division of Forestry and Wildlife Protected Wildlife Permit WL19-23 and United States Geological Survey Bird Banding Laboratory permit 23600. Laboratory work was performed under animal care and use protocols approved by the Institutional Animal Care and Use Committee at the University of California in Santa Cruz, USA (Kilpm2003) and by the Institutional Animal Care and Use Committee at the Smithsonian National Zoological Park (NZP-IACUC Proposal #15-18). Other permits included the US Fish and Wildlife Service Migratory Bird Scientific Collection Permit (MB67895B), United States Department of the Interior Bird Banding Laboratory (permit #21144), Hawai‘i State Protected Wildlife Research Permit (WL 17-08), and Hawai‘i State Access and Forest Reserve Special Use Permit.

Examination of blood smears

One of the authors (C.M. Seidl) examined blood smears on slides for malaria and estimated parasitemias for 299 samples from 141 wild-caught birds within 14 species and 158 samples from 33 experimentally infected birds (two species) by scanning ~50 microscope fields at 1000x magnification using oil immersion and a DM1000 (Leica, Wetzlar, Germany) light microscope and counting the number of red blood cells infected with any stage of *P. relictum.* We estimated the total number of cells examined by counting the number of red blood cells in three randomly selected fields and multiplying the average of these three numbers by the total fields viewed. We examined an average of 5,509 cells per slide (median 5,000; range 800-20,000).

Examining more red blood cells would have increased the probability of detecting infection, and decreased the uncertainty of parasitemia estimates, but would have required additional time, and would not have altered the relationship between parasitemia estimated by qPCR and microscopy.

qPCR quantification

We extracted DNA from avian blood samples with a Qiagen DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) following the manufacturer's protocol for the Purification of Total DNA from Animal Blood and nucleated red blood cells. We quantified the concentration of genomic DNA with a Qubit fluorometer (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) and normalized samples to a starting concentration of 2 ng/µL. The infection status of blood samples was quantified using a qPCR assay with a hydrolysis probe optimized for the GRW4 lineage of *P. relictum* found in Hawai‘i (Beadell et al., 2006) as previously described (Videvall et al., 2021; Neddermeyer et al., 2023; Paxton et al., 2023). The primers used for the assay are adapted to target the cytochrome *b* region (Zehtindjiev et al., 2008), and to date, only one lineage of *P. relictum* has been documented in Hawai‘i (Beadell et al., 2006). We tested each sample in duplicate or triplicate and averaged the cycle threshold (Ct) scores across runs. We considered the result to be a positive detection for *Plasmodium* if a run crossed the threshold baseline within 40 cycles. Samples with one positive run were considered positive and assigned the single run score.

Data analysis

We performed all analyses using R 4.2.0 (R Core Team, 2022). A generalized linear mixed effects model with a binomial distribution and a logit link was used to examine the relationship between parasitemia using microscopy (fraction of cells infected) as the response variable and the qPCR Ct score as the predictor variable. In this model each blood cell examined was an individual data point, and sample ID (a blood sample from a bird on a given day) was included as a random effect to account for the grouping of multiple cells in each sample. We used the *glmer()* function to fit the model and the *bootMER()* function to estimate standard errors for new predictions; both are in the *lme4* package (Bates et al., 2015).

Our goal was to estimate the fraction of infections that may have been missed in a large previous study that examined infection in 2,206 bird samples from eight species using only microscopy (van Riper et al., 1986). We had intended to use the fitted model described above (Fig. 1) to estimate the probability of detecting infection (yes/no) by microscopy as a function of the qPCR Ct score. However, the predictions from this model showed some deviations from the detection (yes/no) of malaria on our slides (Fig. 2; black points and dashed red line, labeled Cell model 5.5 K cells - 5,500 cells were examined; see below). So, we fitted a second model with the detection of infection (yes/no) by microscopy on an entire smear (rather than an individual cell) as the response variable, and the qPCR Ct score as the predictor using logistic regression (a generalized linear model with a binomial distribution and a logit link) for the same set of samples (Figure 2, black dashed line, labeled Smear model,

5.5 K cells). We compared the fit of this Smear model and the Cell model by Akaike Information Criteria (AIC). Because we found (see Section 3) that the model fit to the presence/absence of infection on smears (the Smear model, 5.5 K cells) was a much better fit than the model fit to infections in individual cells (i.e., the parasitemia), we used the Smear model to estimate the fraction of infections missed by microscopy. To re-examine patterns of infections in the historical study that used microscopy (van Riper et al., 1986), we had to account for differences in the average number of cells examined in our study (5,500 cells per slide) and the previous study (~25,000 cells per slide). We adjusted the likelihood of detecting infection by calculating the difference in the likelihood of detection when viewing 5,500 cells versus 25,000 cells using the Cell model. The higher number of cells examined was equivalent to a shift in the detectable Ct scores of 1.8 cycles (see Fig. 2, Cell model 5.5 K and Cell model 25 K), and accounts for the increased detection probability with the number of cells viewed (Valkiūnas et al., 2008). We used this to create an adjusted Smear model (Figure 2, black solid line, labeled Smear model, 25K cells) that estimates the probability that an infection would be detected in each species by microscopy when examining 25,000 cells, and used the qPCR Ct scores from our more recent sampling of wild-caught individuals from that species.

For each species, we estimated the fraction of infections that would be detected by microscopy by drawing from a Bernoulli/binomial distribution with a probability of detection based on the Smear model, 25 K cells, for each qPCR-positive sample and divided the sum of these values by the total number of qPCR-

positive samples to calculate the fraction of infections detected by microscopy. We repeated this set of binomial draws for each species 1,000 times to estimate the uncertainty in the fraction of qPCR-positive samples that would have tested positive by microscopy in our analyses. The inverse of this fraction was the ratio of missed infections when using only microscopy. We used this ratio to estimate the likely infection prevalence for eight species that would have been obtained in the previous study (van Riper et al., 1986) had qPCR been available at that time. We note that this assumes that the parasitemias (but not prevalences) of infected birds from each species are similar between our sample of infected birds from 2015–2022 and the birds captured in the previous study (van Riper et al., 1986).

Finally, we used our parasitemia data from wild-caught birds to test the hypothesis that mist net sampling techniques undersample individuals with high parasitemias. We did this for *Chlorodrepanis virens* which we sampled in the wild and for which experimental infection studies have been published (van Riper et al., 1986; Atkinson et al., 2000, 2001). *C. virens* are frequently infected in their first year, have lower likelihood of infection as adults, and then remain chronically infected for life (Jarvi et al., 2002; Atkinson and Samuel, 2010; McClure et al., 2020). We classified wild-caught birds in our dataset into "high parasitemia" and "low parasitemia" using parasitemia thresholds drawn from the experimental infection studies in which the parasitemias of newly infected birds were tracked over time and encompassed the initial period when parasitemias are highest, peak, and decline (high parasitemia) to below a threshold level that they do not rise above for the remainder

of the study (low parasitemia). We found two parasitemia thresholds that could be used to separate the high parasitemia and low parasitemia periods of infection: 1% and 0.5%, which occurred 47 and 67 days after infection, respectively, for *C. virens* (Figure S1). The high parasitemia periods are five days less than these values because parasites are usually not detectable in these species until day five after infection. We determined the Ct score corresponding to 1% and 0.5% parasitemias using our fitted model which predicts parasitemia by microscopy from qPCR Ct score (See above methods). We then estimated whether the fraction of qPCR-positive birds caught with high parasitemias was less than what we would expect. The expected fraction was the ratio of the length of the high parasitemia period, *H*, to the lifespan of an infected bird, *L*, both in days: H/(L+H). We compared the expected and observed fractions using a binomial distribution with the *binom.test()* function in R. The average life expectancy, *L*, of infected adult birds was calculated, assuming constant type II survival as adults, as 365/(1 - adult annual survival) or 961 days for *C. virens* (Kilpatrick et al., 2006).

Results

Of the 299 samples we tested for malaria by both microscopy and qPCR, we detected *P. relictum* in 36% (109/299) of samples by microscopy and 72% (216/299) of samples by qPCR. Seven samples were positive by microscopy (parasitemias 0.005%-0.04%; 6 of 7 had 1 infected cell in 5-20,000 cells) but negative by qPCR, 3 in wild-caught and 4 in experimentally infected birds.

There was a strong and nearly linear relationship between parasitemia measured using microscopy and Ct score on a logit scale between Ct scores of 29 and 22 (Figure 1). For higher Ct scores, an increasing fraction of samples were negative by microscopy, but infections were occasionally detected by microscopy for Ct scores as high as 38.2 (Figures 1 & 2). We used the fitted model to predict the probability of detecting at least one infected cell in 5,500 cells (the average number of cells we examined) by microscopy for a range of Ct scores (Figure 2, dashed red line labeled Cell model, 5.5K cells). We also fit a model to the presence/absence of malaria on slides (detecting at least one infected cell regardless of parasitemia; Figure 2, black dashed line, labeled Smear model, 5.5K cells). Both relationships suggested that detecting infection by microscopy was very high for Ct scores between 22 and 26 and fell below 50% for Ct scores higher than 32 Cts (Figure 2). However, the Cell model (Figure 2 dashed red line, Cell model, 5.5K cells) fit to parasitemias had a much steeper slope than the Smear model fit to the presence/absence of infection (Figure 2, dashed black line, Smear model, 5.5K cells). Although the Smear model would be expected to fit *slightly* better than the Cell model, since it was fit directly to presence/absence data (rather than fit to the parasitemia data with red blood cells as data points), it was, in fact, a much better fit to the data (delta-AIC = 941.5).

We then used the Smear model, adjusted to reflect examination of 25,000 cells, to quantify the probability of detecting infection by microscopy (Figure 2, Adjusted Smear model, solid thick black line; see Methods) for eight species using the mean parasitemias for each species from our qPCR positive wild bird samples (N

= 656). The probability of detecting a qPCR-positive infection by microscopy, ranged from 20–23% for three introduced species with low parasitemias, *Z. japonicus*(N = 284), *Leiothrix lutea* (N = 101), and *Lonchura punctulate* (N= 12), to 44.0% and 68.7% for two native species, *C. virens* (N = 132) and *H. sanguinea* (N=58), with higher parasitemias (Figure 3A).

We used these estimates of the fraction of infections detected by smear to re-examine the infection prevalence of *P. relictum* in the same eight species from a large-scale study conducted four decades earlier when only microscopy was possible (van Riper et al., 1986). In the previous study (van Riper et al., 1986), the prevalence of infection in *Z. japonicus, C. virens* and *H. sanguinea* using microscopy were 0.90%, 7.3%, and 29.2%, respectively (Figure 3B). These historical prevalence estimates suggest that *C. virens* and *H. sanguinea* had 8–32-fold higher infection prevalence than *Z. japonicus* (Figure 3B). However, if the samples had been tested by qPCR, the ratio of infection prevalence of these species would have been much smaller (4–10-fold), because the low parasitemias of most *Z. japonicus* infections resulted in only 21.9% being detectable by microscopy, whereas the fraction of infections detected by microscopy in *C. virens* and *H. sanguinea* were much higher (Figure 3A).

Next, we examined whether mist netting undersampled highly infected *C. virens*. The expected fraction of infected birds with high parasitemias, if the probability of capture did not vary between high parasitemia and low parasitemia stages, was 0.044 and 0.065 for the two thresholds (Table 1). The fraction of wild

birds sampled with high parasitemias was slightly lower than expected , but these fractions were moderately uncertain and the ratio of the observed fraction of birds with high parasitemias caught in mist-nets to the expected fraction overlapped 1 with both thresholds (Table 1). Our power to detect a bias in sampling was low, due to a low expected fraction of birds with high parasitemias (~5%) and only a moderate sample size (132 qPCR positive birds).

Discussion

The ability to accurately measure infection patterns depends on the sensitivity of the detection method (Valkiūnas and Atkinson, 2020). Innovations in molecular detection and quantification techniques have improved the ability of researchers to detect and quantify parasite loads and have broadened our understanding of parasites as drivers of many ecological and evolutionary patterns (Ricklefs and Fallon, 2002; Asghar et al., 2015). Previous studies have shown that molecular techniques are more sensitive screening and quantification tools for *Plasmodium* spp. infections than microscopy (Jarvi et al., 2002; Valkiūnas et al., 2008; Braga et al., 2011; Ishtiaq et al., 2017). Our study aimed to provide a quantitative framework for integrating historical and contemporary datasets that use qPCR and/or microscopy. We developed a statistical relationship for relating qPCR values to parasitemia values measured by microscopy, using data across three orders of magnitude of infection intensity. We found that the probability of detecting a parasite by microscopy dropped below 50% for infected individuals with Ct scores higher than 32 or parasitemias below

approximately 0.01%. This is primarily due to the fact that most studies only examine 5,000–50,000 host cells because of the time required to examine smears.

Unfortunately, most wild-caught qPCR-positive birds in Hawai‘i had low parasitemias (<0.001%, ≥35 Ct), resulting in microscopy missing a substantial fraction of infections. However, the difference depended on parasitemia, which varied among species, in line with previous studies (Fallon and Ricklefs, 2008; Ricklefs et al., 2018). Infection in several abundant non-native species was underestimated by 4–5-fold, while infection in native birds was only underestimated by 1.5–2.3-fold. The higher detection rates of infection for native species with higher parasitemias has contributed to assertions that Hawaiian honeycreepers are the dominant reservoirs for avian malaria transmission (Atkinson et al., 1995; van Riper et al., 1986; Woodworth et al., 2005; Atkinson and Samuel, 2010; McClure et al., 2020). However, we found that infection prevalence in *Z. japonicus* was much higher than suggested by historical studies done with microscopy; our estimates were similar to those observed in recent studies that used PCR or serology to detect infection (Atkinson et al., 2014; McClure et al., 2020; Neddermeyer et al., 2023). A key question in understanding the role of *Z. japonicus* in *P. relictum* transmission in Hawai‘i is how infectious its low-level parasitemias are to biting mosquitoes. While higher parasitemias likely infect a higher fraction of biting mosquitoes (Pigeault et al., 2015), the relationship between host parasitemia and the fraction of feeding mosquitoes that become infectious is unknown, and is badly needed. More generally, abundant species with mid-level to

lower parasitemias may be important reservoirs, especially in avian communities where native species are absent (McClure et al., 2020).

Our study has several limitations. First, our relationships between Ct scores and parasitemias estimated using microscopy are specific to the lab and equipment where the work was performed and are dependent on template concentrations in the qPCR reactions (i.e., we used a concentration of 2 ng/µL per reaction), primarily because we lack a standard curve for normalizing Ct scores with other labs. This is an important step in future work. Second, we do not yet know the relationship between parasitemia and infectiousness to biting mosquitoes, so we cannot translate parasitemia into infectiousness and determine the relative contributions of avian species to *P. relictum* transmission. Third, the difference between the slopes of our Cell model fit to parasitemia data and our Smear model fit to the presence/absence of infection suggested the distribution of parasitized red blood cells on smears was aggregated (Davidson, 1958; Godfrey et al., 1987), and this made it more difficult to detect infection in highly parasitized smears and accurately estimate parasitemias by microscopy. Fourth, our sample size of qPCR-positive *C. virens* individuals was too low to precisely estimate the fraction of birds caught in mist-nets with high parasitemias, so we could not conclusively determine if parasitemias measured using birds caught in mist-nets are a biased subset of infected birds. Given the low expected fraction of high parasitemia birds, we would need several hundred qPCR-positive individuals of each species to address this question conclusively. Finally, our revised estimates for infection rates from the historical study (van Riper et al., 1986) use

parasitemia data collected between 2015-2022 and assume similar parasitemia levels over time, which might change through the evolution of resistance in avian hosts or changes in *P. relictum*.

Conclusion

PCR-based methods have become the standard for investigating infection prevalence for *Plasmodium spp.* parasites (Ricklefs and Fallon, 2002; Fallon et al., 2003; Ishtiaq et al., 2017). We have created a tool for integrating data from qPCR and microscopy and shown that molecular methods are critical for accurately comparing infection prevalence among species. Several species of introduced birds in Hawai‘i had much higher infection prevalence than historical studies suggested and might play an important role in transmission. Although microscopy is useful for characterizing parasite life stages (especially mosquito-infecting gametocytes), host blood cell counts, and is an inexpensive and quick method for determining parasitemia for high-intensity infections (Godfrey et al., 1987; Valkiūnas et al., 2008), it is insensitive for low parasitemias and produces misleading patterns of infection prevalence. Accurately and more fully characterizing host-parasite interactions requires either a combination of qPCR and microscopy, or multiple qPCR assays that detect different malaria life stages (Wang et al., 2015; Gadalla et al., 2021).

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Tables & Figures

Table 1. Expected and observed probability of capturing birds with high parasitemia *Plasmodium relictum* (lineage GRW4) infections using passive mist net capture techniques. *N* is the sample size of infected birds and *H* is the length of the high parasitemia period. Acute parasitemia thresholds and the length of the high parasitemia period are derived from an experimental infection study conducted by Atkinson et al., 2001. The expected fraction is the ratio of *H* to the lifespan of an infected bird, *L*, both in days: H/(L+H). *L* is derived from Kilpatrick et al., 2006. The expected and observed fractions are compared using a binomial test.

Species	Acute parasitemia threshold (%)	*H* (days)	*N*	Expected fraction	Observed fraction (95% CI)	Observed/expected (95% CI)	*P*-value
Chlorodrepanis virens	1	42	132	0.044	0.030 (0.0083, 0.076)	0.69 (0.19, 1.7)	0.67
C. virens	0.5	62	132	0.065	0.038 (0.012, 0.086)	0.59 (0.18, 1.3)	0.29

CI, confidence interval
P-value ≤ 0.05 considered statistically significant to reject the null hypothesis

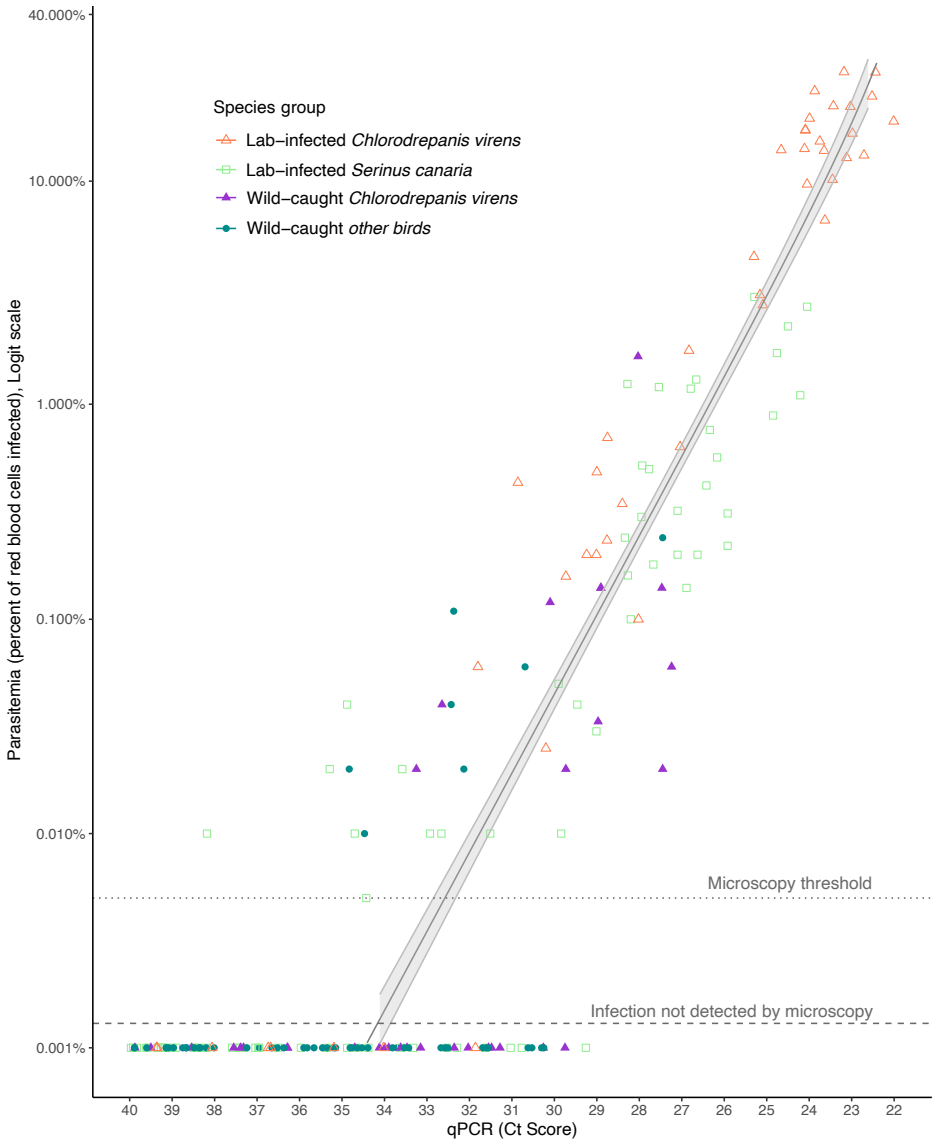

Figure. 1. Relationship between *Plasmodium relictum* parasitemia, quantified by microscopic examination of blood smears and quantitative PCR (qPCR) cycle threshold (Ct) score for 210 blood samples. Point shape and fill indicates the bird species (USA) or species group and the source of *P. relictum* infection (i.e., laboratory infection or wild-exposure). The line shows the fitted model and 95% confidence intervals (Logit (Parasitemia) = 17.78 - 0.85 (S.E. = 0.034)*Ct score, N=210, Z = -25.2, $P < 2 \times 10^{-16}$; random effect standard error: 1.11). Samples with a Ct score ≥ 40 were considered negative and are not shown. The dotted line indicates the lowest microscopy detection threshold for our study (one parasite in 20,000 red blood cells). qPCR-positive samples for which infection was not detected by microscopy are shown below the dashed line and given a parasitemia of 0.001% to facilitate visualization.

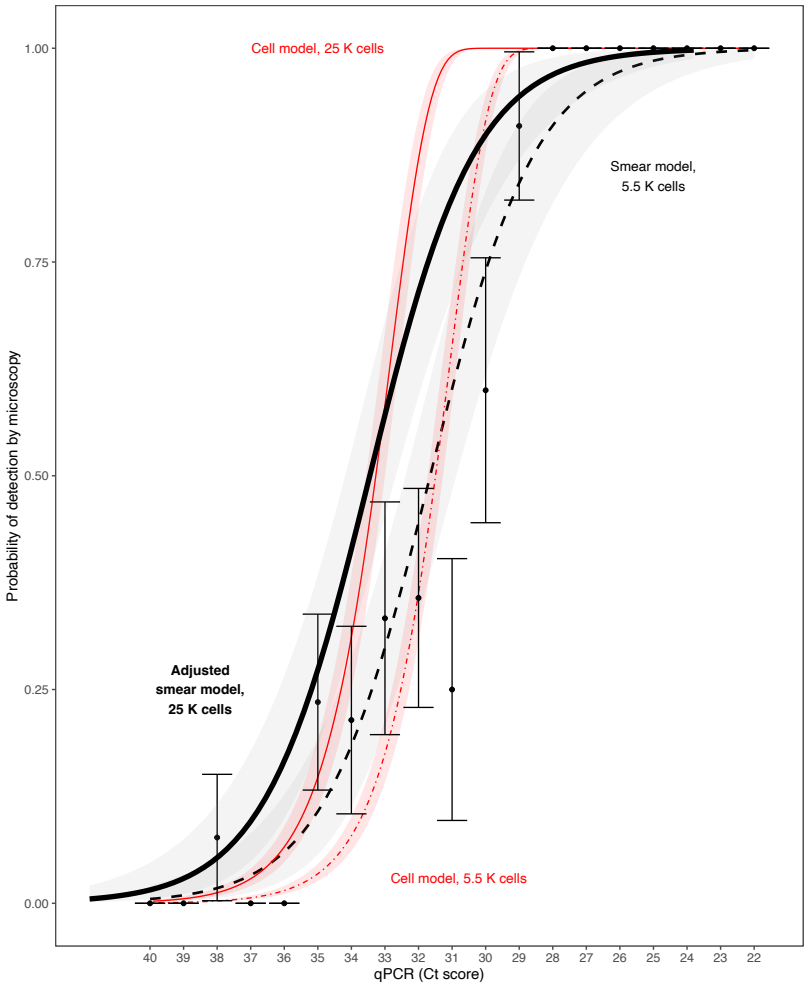

Figure 2. The probability of detecting a *Plasmodium relictum* infection by microscopy plotted against the quantitative PCR (qPCR) cycle threshold (Ct) score for that sample with four models. Points show the fractions of smears (with a mean of 5,500 cells examined) where at least one infected cell was seen, grouped by rounding the Ct score, and error bars show the binomial standard error of this fraction. The dot and dash line (cell model, 5.5 K) shows the probability of detecting at least one infected cell when viewing 5,500 red blood cells by microscopy using the fitted model in Fig. 1. The solid thin line (cell model, 25 K) shows the probability of detecting at least one parasite cell in a smear by microscopy when viewing 25,000 red blood cells, also based on the fitted model from Fig. 1. The black dashed line, (smear model, 5.5 K cells), shows a logistic regression model fitted to the points in this graph where a blood sample is a point (logit (Fraction of smears positive) = 19.9 – 0.63 (S.E. = 0.085)*Ct score; $N = 210$, $Z = -7.4$, $P = 1.57 \times 10^{-13}$). The solid thick black line (smear model, 25 K cells) is the same as the dashed black line but is shifted 1.8 Ct scores to the left to estimate the probability of detecting infection when 25,000 cells are viewed, rather than 5,500 (see Section 2 for details).

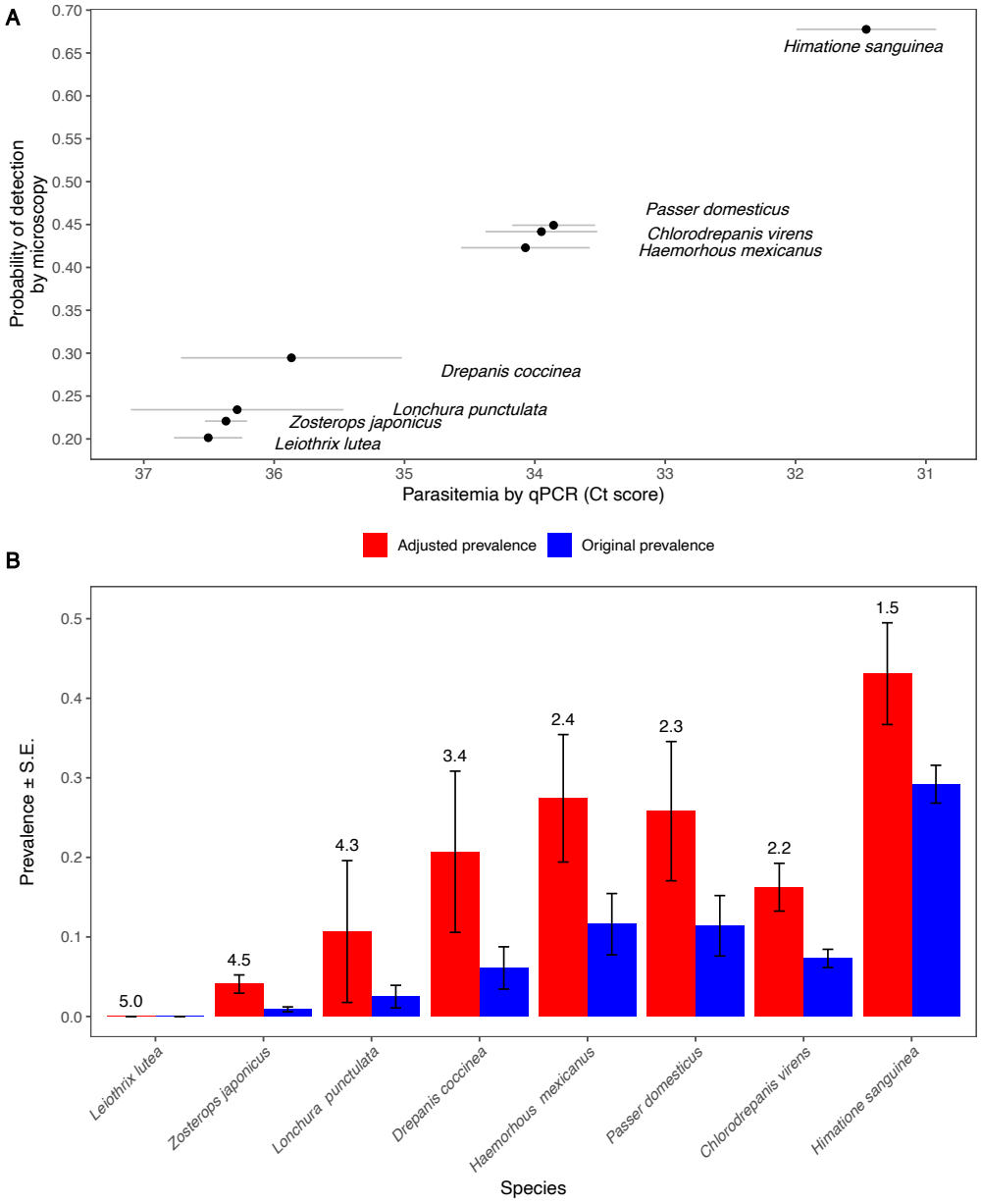

Figure 3. Prevalence of *Plasmodium relictum* infection in eight species of birds in Hawai‘i (USA). **A**) Average parasitemia by quantitative PCR (qPCR) cycle threshold (Ct) score (± S.E.) and detection probability by microscopy when viewing 25,000 cells for each species. Note that higher Ct scores indicate less *P. relictum* DNA. **B**) Infection prevalence measured using microscopy in the same eight species (van Riper et al. 1986) (blue bars) and estimates for prevalence if blood samples had been tested by qPCR (red bars). Numbers above red bars are the ratios of adjusted prevalence by qPCR to the measured prevalence by microscopy fit with the adjusted smear model with 25,000 cells (bold black line) from Fig. 2.

Supplemental materials

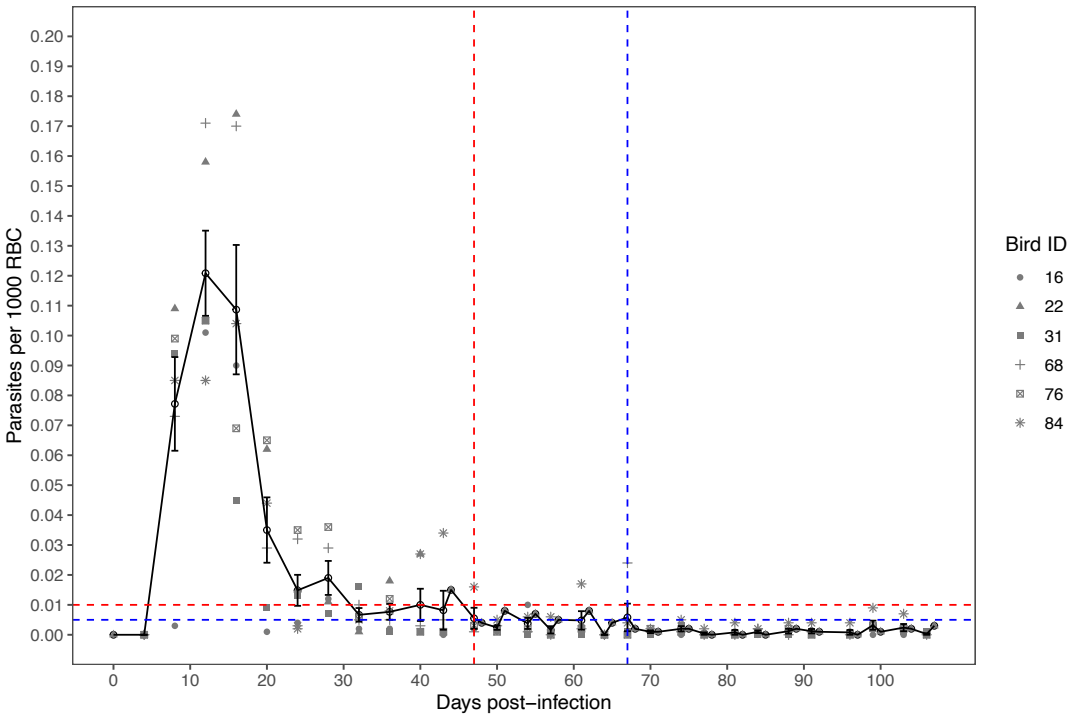

Figure. S1. Mean parasitemia of surviving experimentally infected birds over time and thresholds dividing "high parasitemia" and "low parasitemia" periods at 1% (dashed lines) and 0.5% (dotted lines) for *Chlorodrepanis virens.* Data are mean parasitemia (open circle) with standard error bars and parasitemias of individual birds (Bird ID) in gray. Parasitemia data presented this figure are sourced from Atkinson et al., 2001 *J Wildl Dis*.

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CHAPTER 3

Infectiousness of native and non-native bird species for avian malaria in Hawai‘i

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Abstract

Multihost vector-borne diseases are responsible for a significant fraction of human and wildlife infectious disease burdens. Identifying which host species infect vectors is essential for understanding pathogen transmission. For one of the world's most well-studied multi-host pathogens, avian malaria (*Plasmodium relictum*), the role of many species in transmission is unknown. We used domestic canaries (*Serinus canaria*) experimentally-infected with *P. relictum* (lineage GRW4) and exposed to Hawaiian *Culex quinquefasciatus* mosquitoes to quantify the relationship between host parasitemia (fraction of red blood cells infected) and mosquito infectiousness across a range of parasitemias, temperatures, and time since feeding. We used the relationship to estimate the infectiousness of 17 species of birds in Hawai‘i using 743 parasitemia estimates from wild-caught birds. Mosquito infectiousness (the fraction

of blood fed mosquitoes with disseminated *P. relictum* infections) increased with host parasitemia, temperature, and the time since feeding, with stronger effects of parasitemia at higher temperatures. The relationship between mosquito infectiousness and parasitemia was gradual, not sharp, resulting in a wide range of parasitemias being partly infectious to mosquitoes. In addition, parasitemia varied substantially within bird species. The combination of a gradual relationship and high within-species variability resulted in considerable overlap between the infectiousness of native and non-native birds. Furthermore, we found that the low parasitemia but long-lasting chronic infection stage of *P. relictum* hosts is likely more important for transmission than the high parasitemia but short acute stage for certain species. These results provide a framework for determining the role of species in transmission of avian malaria in Hawai‘i. They also demonstrate the importance of within-species variability in pathogen load and of quantifying the steepness of the relationship between pathogen load and vector infectiousness.

Introduction

Transmission of multi-host vector-borne pathogens depends on community composition because hosts vary in their abundance, infectiousness, and contact rates with vectors (Komar et al. 2003; LoGiudice et al. 2003; Kilpatrick et al. 2006a). The infectiousness of different host species is needed to predict how changes in community composition will influence transmission (LoGiudice et al. 2003; Kilpatrick et al. 2006a; McClure et al. 2020). However, determining host

infectiousness can be challenging and requires both experimental infection studies of wild animals, which are logistically difficult, and measuring the infectiousness of host pathogen loads to a biting vector. As a result, the role of different species in the transmission of avian malaria (*Plasmodium relictum*), which is one of the world's best-studied multi-host pathogens, is unknown in many communities.

The shape of the relationship between pathogen load and vector infectiousness can vary across pathogens, strains, and vector species and populations (Turell et al. 2001; Tiawsirisup et al. 2005; Kilpatrick et al. 2010; Cecilia et al. 2022). The steepness of the slope, the location of the threshold, and the range of pathogen load variation within species determine the infectiousness of species with lower pathogen loads, or in the case of malaria species, parasitemias (precent of red blood cells infected) and infectiousness to mosquito vectors. For example, when there is little within-species variation in parasitemia (Figure 1, parasitemias under short green and purple rectangles), a relationship with a steep slope and high threshold (Figure 1, black line A) leads to only higher parasitemia hosts infecting biting mosquitoes; species with low parasitemias will be dead-end hosts. In contrast, if the relationship's slope is steep and the threshold is low (Figure 1, red line B), all high parasitemia species and most low parasitemia species will infect mosquitoes. A shallow slope relationship (Figure 1, blue line C) allows for both high and low parasitemia species to be partly infectious. Finally, if within-species variation in parasitemia is large (Figure 1, long green and purple rectangles) then some individuals of both high and low parasitemia species will be partly (blue line C) or almost completely infectious

(red line B or black line A). Thus, the slope of the relationship, the location of the threshold, the mean parasitemias for each species, and the range of parasitemias within each species will all determine the role a species plays in transmission. The relationship-dependent role of "high" and "low" parasitemias in transmission also applies to comparisons between the high parasitemia acute phase of infection that is experienced by individuals during the first days to weeks of infection and the low parasitemia chronic phase of infection that follows and lasts for months to years with some parasite species.

Transmission of vector-borne diseases is also strongly influenced by time and by temperature, which alters both vector and pathogen traits (Purse et al. 2005; Kilpatrick et al. 2008; Paaijmans et al. 2013; Ciota et al. 2014; Kraemer et al. 2015, Seidl et al. 2024). Ambient temperature influences invertebrate resistance to infection for many pathogens (Kobayashi et al. 1981; Reynolds et al. 2003; Stacey et al. 2003; Murdock et al. 2014) and pathogen replication rate (Chen & Shakhnovich 2010). As a result, the probability and timing of disseminated infection and salivary gland invasion in mosquitoes is temperature-dependent (Vanderberg & Yoeli 1966; Dohm et al. 2002; Reisen et al. 2006). Thus, to understand how transmission of avian malaria is likely to change across elevations that differ in host community composition and in temperature, it is necessary to quantify the relationships between host infectiousness and parasitemia across a range of temperatures.

Mosquito-borne diseases have contributed to the decline and extinction of many species of Hawaiian birds (Warner 1968; LaPointe et al. 2012). Recent

dramatic declines in Hawaiian native bird populations are associated with increases in avian malaria (*P. relictum*, lineage GRW4) transmission in high-elevation habitats as a result of with climate change (Paxton et al. 2016, 2022). Infection in Hawaiian honeycreepers causes high parasitemias, and high levels of mortality (van Riper 1991; Atkinson et al. 1995; Yorinks & Atkinson 2000). The high parasitemias measured in native birds have led to hypotheses that they are the primary reservoirs infecting mosquitoes with avian malaria (van Riper et al. 1986; Pigeault et al. 2015). However, more than 50 non-native bird species are established in the main Hawaiian islands and most can be infected with avian malaria (Long 1981; Moulton & Pimm 1983; van Riper et al. 1986; Pratt et al. 1987; Atkinson et al. 2014; Neddermeyer et al. 2023). Avian malaria transmission occurs in communities entirely composed of non-native species, suggesting that the non-native species that co-occur with native birds may also contribute to transmission (McClure et al. 2020; Neddermeyer et al. 2023). Non-native species are hypothesized to have lower parasitemias than native species (van Riper et al. 1986; Atkinson & Samuel 2010); however, few parasitemia data exist for most species. As a result, the role of many birds in avian malaria transmission is unknown.

A critical component for identifying the role of different species in avian malaria transmission in Hawai‘i is determining the relationship between host parasitemia and infectiousness to biting mosquitoes. Previous work has quantified the parasitemia and infection prevalence in many native species (Atkinson et al. 1995, 2000; Yorinks & Atkinson 2000; Samuel et al. 2015; McClure et al. 2020), and while

parasitemia data for some non-native species exist (van Riper et al. 1986), the infectiousness of most parasitemias to *Cx. quinquefasciatus* vectors is unknown. A few studies have compared the fraction of mosquitoes that are infected after feeding on birds with different parasitemias, but these studies either measured mosquito infection in terms of oocyst prevalence and burden inside the mosquito's midgut (Pigeault et al. 2015), which does not always predict transmission potential (LaPointe et al. 2010; Graumans et al. 2020), or they categorized host parasitemias into a broad high or low category (Gutiérrez-López et al. 2019). Furthermore, a mean parasitemia estimate may not sufficiently capture a species' role in transmission, especially when large variation in parasitemias exists among individuals within a species (Figure 1). Depending on the slope of the relationship between parasitemia and mosquito infectiousness, few or many individuals will play a role in transmission (Figure 1). Understanding when and which individuals, species, and species compositions are infecting mosquitoes is key for understanding their roles in avian malaria transmission across Hawai‘i.

Our goal was to determine the role of species in transmission of avian malaria in Hawai‘i, and how this might vary across temperature gradients, by quantifying the relationship between host parasitemia, ambient temperature, and time since feeding on infectiousness of *Cx. quinquefasciatus*. We derived this relationship by feeding *Cx. quinquefasciatus* on domestic canaries (*Serinus canaria*) infected with *P. relictum* lineage GRW4 collected from Hawai‘i . We used the estimated mosquito infectiousness of different species' parasitemias and species composition of eleven

host communities to illustrate how these estimates could be used both to predict the infectiousness of a whole host community as well as individual species contributions to it. Additionally, we provide a comparison of infectiousness between the acute and chronic stage of host avian malaria infection to assess the relative contribution of each stage to transmitting mosquitoes on the landscape.

Materials and methods

Plasmodium relictum and Culex quinquefasciatus collection and maintenance

We collected three isolates of *Plasmodium relictum* (lineage GRW4) from wild birds at three sites on Hawai‘i Island, an ‘apapane (*Himatione sanguinea*) from Pu’u Wa’awa’a Forest Reserve (ISO 2: 19.738154°, -155.875234°, 1,230 m above sea level) in February 2020, a House Sparrow (*Passer domesticus*) from Kapoho, Hawai‘i (ISO 1: 19.498176°, -154.853154°, 63 m above sea level) in April 2020, and a Warbling White-eye (*Zosterops japonicus*) from Captain Cook, HI (ISO 3: 19.461278°, -155.896500°, 204 m above sea level) in April 2023 (Figure S1). We took 50–100 µl of blood from the brachial vein of birds and placed it into a 1 mL syringe with an appropriate volume of citrate–phosphate–dextrose solution with adenine (CDPA; Sigma-Aldrich, St. Louis, MO, USA) to create a 1:9 ratio (Carlson et al. 2016) and stored it up to 48 hrs at 4 °C. We also used 1–2 µl of blood from the same bird to create a thin blood smear that was air-dried for 30 minutes, fixed in 100% methanol, and later stained for 1 hr. with Giemsa (Valkiūnas 2005). We screened each stained smear by examining 50 microscope fields at 1000x

magnification using oil immersion to determine the presence and parasitemia of a sample. If we observed one or more cells infected with malaria, we inoculated the blood-CDPA mixture intramuscularly into the pectoral muscle of a domestic canary. We passaged isolates one to five times in canaries via intramuscular inoculations of 50–100 µl of infected blood between birds before exposure to mosquitoes in feeding trials (see below), or before cryopreservation in glycerolyte for future use (Moll et al. 2013). Most isolates used in mosquito feedings had two passages between the wild source bird and the bird used for mosquito feedings. All work with wild and laboratory birds was performed under animal care and use protocols approved by the Institutional Animal Care and Use Committee at the University of California in Santa Cruz, USA (Kilpm2003).

We reared *Culex quinquefasciatus* Say (Diptera: Culicidae) mosquitoes at 26 °C in 70–80% humidity under a 12L:12D hr. photoperiod from egg rafts collected at three locations on Hawai‘i Island between 2020–2023. These included two sites near Hilo (MC2: 19.685787, -155.080147°, 88 m above sea level in April 2020; and MC1: 19.7646407, -155.0924556°, 55 m above sea level in March 2022), and a site near Captain Cook (MC3: 19.4613276, -155.8981797, 205 m above sea level in March 2022 and 2023; Figure S1). Egg rafts were collected and shipped within 24 hrs. of laying to Santa Cruz, California. Upon arrival, rafts were immediately floated in plastic pans (44 cm × 25 cm × 10 cm) filled with 1 L of deionized water and hatched larvae (200–350 per pan) were fed daily 0.2–0.4 g of ground fish food (Koi’s Choice® Premium Fish Food). Pupae were transferred to mosquito cages (30 cm^3,

BugDorm) in the same incubator, and emerging adults were fed ad libitum on 10% sucrose solution-soaked cottons. Seven to 14-day-old wild-collected, F1, and F2 generation adults were used in experimental feedings. All mosquitoes were sucrose-starved for 48 hrs. prior to blood feeding. To create F1 and F2 generations, blood-fed mosquitoes were provided a small cup of water for egg laying starting two days after blood feeding. Cups were checked daily for egg rafts.

Experimental infection and mosquito feeding

We inoculated canaries intramuscularly with 50–200 µL of 0.1–4.25% infected whole blood from a single avian malaria isolate. Infected blood was either fresh, containing an isolate passaged 1–4 times in other canaries, or a thawed deglycerolized sample. Starting on day 5 post-infection and every 3 days after, we took 5–10 µL of blood by brachial venipuncture and screened blood using both thin blood smears (described above) and quantitative PCR (qPCR) (Paxton et al. 2023) to detect infection and estimate parasitemia. Once parasitemia loads were in the range observed in birds in Hawai‘i (qPCR Ct scores: 20–40, Parasitemia: 0.00000438–0.56 infected cells/red blood cell; see below), we placed birds, unrestrained, inside a vertical PVC cylinder (10 cm diameter x 30 cm height) containing a wooden perch. The cylinder was elevated on a wire platform and capped with plastic mesh (Figure S2). This apparatus allowed mosquitoes to access birds from above and below but prevented birds from flying. The apparatus was placed inside a BugDorm mosquito cage with 100 female mosquitoes. Birds and mosquitoes were held together overnight

from 2000 to 0600 hr. (10 hrs.) in a room at 24 °C. We collected fully engorged mosquitoes, divided them into four groups, and transferred the groups into incubators set to 18, 20, 24, or 28 °C. We held mosquitoes within each incubator until their dissection dates (see below).

Feeding trials and mosquito assays

We collected a subset of mosquitoes from each incubator at three time points that varied depending on the temperature of the incubator. We chose time points with the potential to capture the rise in the fraction of mosquitoes with disseminated infections over time (LaPointe et al. 2010). Our assay days, post-feeding, were 5, 8, and 12 d for 28 °C; 6, 10, and 14 d for 26 °C; 12, 17, and 23 d for 20 °C, and 22, 33, and 44 d for 18 °C. Mosquitoes that died between testing days were also dissected but not assayed. All mosquitoes were dissected by cleanly separating the thorax from the abdomen at the scutellum with sterile dissection needles. We placed the abdomen in one 1 mL vial containing 0.5 mL of 70% ethanol and placed the head, thorax, and any legs in a second vial that we used to test for a disseminated infection. For a subset of mosquitoes, we collected salivary secretions before dissection using a modified *in vitro* capillary transmission assay (Miller et al. 2021). We placed a live mosquito's proboscis into a 50 µL capillary tube (Thermo Fisher Scientific, Waltham, Massachusetts, United States) filled with 25 µL of immersion oil. After 45 minutes, the contents were expectorate into a 1.5 mL Eppendorf tube with 0.5 mL of Queen's

Lysis Buffer (Seutin et al. 1991). All samples were stored for 1–90 days frozen at -20 °C before using qPCR to detect P. relictum DNA.

Bird sample collection and censuses

We collected blood samples from 36 species of wild birds on the islands of Kaua'i, Oahu, Maui, and Hawai‘i (Hawai‘i, USA) between 2015–2022. Birds were captured in forested sites between 29–2,000 m above sea level using 6, 9, and 12 m long 38-mm mesh mist nets. From each individual, we drew 25–100 µL of blood by brachial venipuncture and placed it in 1 mL of Queen's Lysis Buffer (Seutin et al., 1991). We stored samples at room temperature for 1–90 days or frozen at -20 °C before using qPCR to quantify parasitemia (see qPCR analysis below).

We conducted unlimited-distance point counts at 11 sites to determine the species composition and relative abundance of bird communities. We conducted four point counts, separated by at least 200 m, at each site between 0600 and 1000 hrs. from February to June 2020. We quantified relative abundance as the fraction of all detections at all four points of a site made up of each species. We conducted fieldwork under Hawai‘i Division of Forestry and Wildlife Protected Wildlife Permits (WL19-23; WL 17-08), USGS Bird Banding Laboratory permit numbers (#23600, #21144), and a Hawai‘i State Access and Forest Reserve Special Use Permit.

qPCR analysis

We extracted DNA from avian blood and mosquito samples using a Qiagen DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) following the manufacturer’s

protocols for the Purification of Total DNA from nucleated red blood cells and from saliva. To process mosquito abdomens and thoraxes/legs/heads, we modified the kit protocol to include a bead-beating step prior to overnight lysis. Bead beating was performed at 2000 strokes/min for 2 minutes using a GenoGrinder Mini tissue homogenizer. We quantified the concentration of genomic DNA with a Qubit fluorometer (Invitrogen) and normalized samples to a starting concentration of 2 ng/μL. We quantified the infection status of blood and mosquito samples using a qPCR assay with a hydrolysis probe optimized for the GRW4 lineage of *P. relictum* found in Hawai‘i (Beadell et al. 2006), as previously described (Paxton et al. 2023). The primers used for the assay are adapted to target the cytochrome b region (Zehtindjiev et al. 2008), and to date, only one lineage of *P. relictum* has been documented in Hawai‘i (Beadell et al. 2006). We tested each sample in duplicate or triplicate and averaged the cycle threshold (Ct) scores across runs. We considered the result to be a positive detection for Plasmodium if a run crossed the threshold baseline within 40 cycles. Samples with one positive run were considered positive and assigned the single run value.

Data analysis

We examined the fraction of blood fed mosquitoes: infected (# positive abdomens/# fed), with disseminated infections (# positive in thorax, head, and legs/# fed) and transmitting (# positive salivary secretions/# fed). We focused our temperature and parasitemia analyses on the fraction of mosquitoes with disseminated

infections because we only collected salivary secretion data from a subset of mosquitoes. We used a generalized linear model with a binomial distribution and a logit link to examine the effect of parasitemia, temperature, and days post-feeding and their interactions on the fraction of mosquitoes with disseminated infections. To estimate parasitemia for infections that were too low to accurately quantify by blood smear (<0.01%), we used the qPCR Ct value and a relationship between Ct value and parasitemia to estimate the parasitemia (Seidl et al. 2023). We used the fitted model to predict the fraction of mosquitoes that would have disseminated infections across a range of parasitemias at each temperature. We estimated the fraction of mosquitoes that would transmit malaria by multiplying the predicted fraction of mosquitoes with disseminated infections for a given parasitemia, temperature, and day by the fraction of mosquitoes with disseminated infections that also had qPCR-positive salivary secretions. The salivary secretion data were averaged across all temperatures, days and parasitemias; there was insufficient data to estimate this probability independently as a function of these three factors.

We estimated avian malaria host infectiousness, or the fraction of mosquitoes that would have disseminated infections after feeding on a parasitemia, for wild bird samples using the fitted relationship from our canary experimental infections studies of *Cx. quinquefasciatus* held at 24 °C and tested 10 days after feeding. The relative values of host infectiousness were similar for other values of temperature and time since exposure. We tested whether native and non-native bird species differed in parasitemia and in infectiousness using a generalized linear mixed effects model with

a beta distribution and species as a random effect using the glmmTMB package (Brooks et al. 2023).

We estimated the infectiousness of different bird communities by multiplying our estimates of relative species abundance from point counts with our estimates of each species' average infectiousness. This calculation assumes that mosquitoes feed on species in proportion to their abundance because studies examining non-random feeding have yet to be published. For species without parasitemia data or positive sample sizes less than 5, we used estimates from the nearest taxonomic group (Genus, Family, Order or Class, after averaging empirical data at the same taxonomic level to generate a mean).

Finally, using the mosquito infectiousness and parasitemia relationship, we examined whether the early, high parasitemia acute stage of infection produces more infectious mosquitoes than later, lower parasitemia chronic stage for Hawai‘i ‘amakihi (*Chlorodrepanis virens*) and ‘apapane (*Himatione sanguinea*), which are abundant and important hosts in many native forest habitats (Ralph & Fancy 1995; Woodworth et al. 2005; Samuel et al. 2015; McClure et al. 2020). These species are well-sampled in the wild and have published experimental infection studies (van Riper et al., 1986; Atkinson et al., 2000, 2001). Hawai‘i ‘amakihi are frequently infected in their first year, have lower likelihood of infection as adults, and then remain chronically infected for life (Jarvi et al. 2002; Atkinson & Samuel 2010; McClure et al. 2020). Capture data suggest ‘apapane infections are similar (Samuel et al. 2015). We used a parasitemia threshold of 1% to separate high and low

parasitemia stages (Seidl et al. 2023). For the high parasitemia stage, we determined the infectiousness of the average parasitemia over the duration of the high parasitemia stage before the 1% parasitemia threshold. In Hawai‘i ‘amakihi the high parasitemia stage covered the first 42 days of infection and in ‘apapane, it covered the first 60 days of infection (van Riper et al. 1986; Atkinson et al. 2000, 2001; Seidl et al. 2023). For the low parasitemia stage, we calculated the infectiousness of the average parasitemia for the days following the 1% parasitemia threshold. The duration of the low parasitemia phase was the lifespan of an infected Hawai‘i ‘amakihi which, assuming constant type II survival as adults, is 365/(1 - adult annual survival) or 961 days (Kilpatrick et al. 2006b), minus the number of days in the high parasitemia stage. For ‘apapane, we estimated their lifespan and low parasitemia phase to be 1217 days (Samuel et al. 2015). We assumed 10 mosquitoes fed on each host each day since the start of infection and these mosquitoes were transmitting after 10 days at a temperature of 24 °C. We then compared the total number of mosquitoes infected in these two stages by multiplying the number of fed mosquitoes over the duration of each infection stage by that stage’s average infectiousness.

Results

We exposed mosquitoes to 21 parasitemias in canaries ranging from 0.00000438–0.564 parasites per red blood cell and collected data from 820 mosquito thoraxes and 272 salivary secretions. The best fitting model for the fraction of mosquitoes with disseminated infections included parasitemia (Ct value), temperature

and days since feeding and two-way interactions between temperature and parasitemia (Ct value) and temperature and days since feeding (Figure 2, Table S1). The interactions between temperature and both parasitemia and days since feeding resulted in stronger positive effects of both parasitemia and days since feeding as temperature increased (Figure 2). At the coolest temperature (18 °C) the effect of parasitemia on the fraction of mosquitoes with disseminated infections was undetectable, whereas at warmer temperatures the effect was strong (Figure 2; compare the slope of lines in the upper left panel at 18 °C to lower right panel at 28 °C). The effect of days since feeding was strongest at warmer temperatures. For example, a 5-day increase in days since feeding from the earliest testing day resulted in a 66.4% increase in the fraction of thoraxes with disseminated infections in 28 °C but only a 11% increase in 18 °C. Of the 22 mosquitoes with both disseminated infection and salivary secretion samples, 9 or 41% had detectable avian malaria DNA in their salivary secretions.

We tested 2,630 blood samples from 36 bird species; 760 samples (29%) from 27 species (75%) were positive for avian malaria by qPCR. We found that native bird species had higher average parasitemias than non-native birds (Native = 0.12%, Non-native = 0.095%; Table S3, Figures 3A, S4A); however, parasitemias of some native and non-native birds overlapped substantially because of enormous variability in parasitemia within species. For example, parasitemias of native ‘apapane (*Himatione sanguinea*) and Hawai‘i ‘amakihi overlapped broadly with non-native Warbling

White-eyes (*Zosterops japonicus*) and House Finches (*Haemorhous mexicanus*) (Figure 3A).

We used the fitted model (Table S1) to estimate the fraction of mosquitoes that would develop disseminated infections 10 days after feeding on parasitemias (at 24 °C) from 743 positive blood samples from 17 species. The parasitemias of native species produced, on average, slightly higher fractions of disseminated infections than non-native species (Table S4).

We then estimated host infectiousness (fraction of mosquitoes transmitting) for the same samples (Figure 3B). Average host infectiousness for non-native species was 7% (SE ± 0.011, Range: 3% -21%), and for native species, 10% (SE ± 0.02; Range: 4–30%; Figure 3B, S4B). For example, after feeding on a bird with the average parasitemia of a field-caught Hawai‘i ‘amakihi (0.41%), the fitted model suggests that 45% of mosquitoes will develop disseminated infections at 24 °C 10 days after feeding, and 18% will have *P. relictum* DNA in their saliva to transmit malaria (Figures 2 & 3). In comparison, House finch mean infectiousness was 11% and for Warbling white-eyes it is 9%. However, because parasitemias of native and non-native birds overlapped broadly, they also overlapped in infectiousness.

We conducted point counts at 11 sites and recorded 28 species (Figure S1, Tables S5, S6). Warbling white-eye (WAWE) was the most frequently observed species and comprised 9-40% of all detections across all 11 sites (Figure 4A). Hawai‘i ‘amakihi was the most frequently observed native bird and was observed at six sites where it comprised 1–60% of detections (Figure 4A). Other native bird

species detected on point counts included ʻapapane, ʻōmaʻo (*Myadestes obscurus*), and Hawaiʻi ʻelepaio (*Chasiempis sandwichensis*). We detected 24 species of introduced birds from Asia, North America, South America, Europe, and Africa (Figure 4A; Table S6).

We found bird communities had similar community infectiousness but varied in their composition and which species comprised the largest fractions of community infectiousness (Figure 4B). Community infectiousness only varied among sites by approximately 20% (mean = 19.6, SD ± 1.83), ranging from 17.9 in Kapoho to 26.0 at Kahuku. At the sites where they were present, two native Hawaiian honeycreepers (ʻapapane and Hawaiʻi ʻamakihi) made up a large fraction of the community infectiousness. For example, at Kahuku, Hawaiʻi ʻamakihi and ʻapapane made up 91.4% (56.1% and 35.3%, respectively, Figures 4B, S6) of the community's infectiousness. Warbling white-eyes, which were present in all sites, also comprised a substantial fraction of community infectiousness (mean: 19%; SD ± 8%; range 6–36%; Figures 4B, S6). Yellow-fronted canaries and House finches were important species in contributing to community infectiousness as well (Figures 4B, S6).

For native Hawaiian honeycreepers, we found that the low parasitemia phase of infection likely results in many more infections than the high parasitemia phase. For Hawaiʻi ʻamakihi and ʻapapane, we estimated that approximately 17 times more infectious mosquitoes would arise from mosquitoes feeding on them during their much longer low parasitemia chronic stage than would during their short high parasitemia acute phase, despite the higher infectiousness during the high parasitemia

phase (Table 1). This is because the more than 10-fold difference in parasitemia between high and low phases translates to a less than 1.5-fold difference in infectiousness (Table 1).

Discussion

Avian malaria infects numerous bird species in Hawai‘i , and its transmission depends on their parasitemias and the infectiousness of these parasitemias to mosquito vectors. We found enormous within-species variation in parasitemia and a relatively gradual relationship between host parasitemia and infectiousness to biting mosquitoes, with the steepness of the relationship increasing with temperature. This gradual relationship and within-species variation in parasitemia resulted in a broad overlap in the infectiousness of many species. Specifically, the infectiousness of many non-native species overlapped with that of native species. Native birds were on average more infectious than non-native birds, but the relative differences in mean infectiousness were small. Furthermore, the gradual relationship between host parasitemia and infectiousness to mosquitoes (rather than a sharp threshold), elevated the relative importance of low-level chronic infections compared to high parasitemia acute phase infections. We found that chronic infections likely produced many more infectious mosquitoes than the initial, short-lived, high parasitemia stage of infection over the lifespan of two species: Hawai‘i ‘amakihi and ‘apapane.

We found that many species of birds could play an important role in infecting mosquitoes with avian malaria because of a gradual relationship between host

parasitemia and infectiousness to mosquitoes and large within-species variation in parasitemias (similar to Figure 1, line C and broad rectangles). The smaller differences in species infectiousness than previously hypothesized challenges previous assumptions that native Hawaiian birds are the primary hosts contributing to transmission of avian malaria (van Riper et al. 1986). These results suggest that many species and individuals have parasitemias capable of contributing to transmission of avian malaria. In contrast, had we found a steeper relationship between parasitemia and mosquito infectiousness, or less within-species variation in parasitemia, some species may have been effectively dead-end hosts.

We found that Hawai‘i ‘amakihi and ‘apapane likely infect many more mosquitoes in their chronic, low parasitemia stage of infection than in their initial high parasitemia infection stage. This is because the length of the high parasitemia stage is much shorter than the chronic phase relative to the bird’s lifespan (van Riper et al. 1986; Atkinson et al. 2001a; Kilpatrick et al. 2006b) and, importantly, because the parasitemias in the chronic phase were partly infectious to mosquitoes. Low-level parasitemias play a role in the transmission of other malaria species (Okell et al. 2012; Aguas et al. 2018; Andolina et al. 2021), but there has long been considerable uncertainty over the relative roles of chronic vs. acute infections in avian malaria transmission. The much larger importance of the chronic phase of infection in infecting mosquitoes may reduce the need for experimental infection studies which are challenging to conduct. Instead, parasitemia data generated from mist-net

sampling of wild-birds, which are mostly chronically-infected individuals (Seidl et al. 2023), can provide an initial estimate of a species' infectiousness.

Three notable limitations affected this study. First, we were unable to analyze the probability of transmission given disseminated infection, or fraction of mosquitoes with positive salivary secretions, as a function of temperature, days since feeding, or parasitemia. There were insufficient salivary secretions to enable rigorous analyses. In addition, the salivary assay may have produced false negatives if inadequate mosquito saliva was expectorated into the capillary tube, which would lead to underestimates in our calculation of the fraction of transmitting mosquitoes (See Figure S5 for disseminated infection estimates for Figure 3; Gloria-Soria et al. 2022). Previous work on *P. relictum* in Hawai‘i found the prevalence of sporozoites in the salivary glands/prevalence of oocysts was greater than 70% for temperatures above 20 °C but <30% below it (LaPointe et al. 2010), and studies of human malaria, *P. falciparum*, in multiple vectors also found high values (80–100%) at many temperatures (18–28 °C) (Shapiro et al. 2017; Waite et al. 2019; Suh et al. 2023). Second, our calculations of hosts' contribution to community infectiousness assumed that mosquitoes feed on hosts in proportion to their abundance, which is at odds with many studies of mosquito feeding patterns (Kilpatrick et al. 2006a). Finally, we had no parasitemia data for several species that were present at the eleven sites. We estimated the infectiousness of some of these species using taxonomically close relatives, but for a few others we were forced to use a broad average, and some of these species may have been dead-end hosts or refractory to infection (e.g.,

Phasianidae or Columbidae). Understanding how host community composition affects transmission of avian malaria across all communities in Hawai‘i will require studies of mosquito feeding preferences and parasitemia data (or susceptibility data) for all species.

The shallow slope of the parasitemia-infectiousness relationship that we observed helps to explain the presence of avian malaria in fully non-native bird communities in Hawai‘i where most species have relatively low parasitemias (LaPointe et al. 2012; Atkinson et al. 2014; Neddermeyer et al. 2023). The results here suggest that many and possibly most bird communities in Hawai‘i support transmission of avian malaria. Other factors, such as vector survival, and temperature may play larger roles than community composition in determining the distribution of malaria. However, community composition (the density of native species) has been correlated with the fraction of infected mosquitoes and appeared to be more important than vector abundance (McClure et al. 2020), suggesting that community composition is important in modulating avian malaria transmission. We encourage additional work on vector feeding preferences to more clearly define the role of different host species in transmission.

Conclusion

Avian malaria (*P. relictum*) is a globally distributed vector-borne pathogen that infects hundreds of host species (Bensch et al. 2009), limits the distribution of several species and has contributed to multiple species extinctions (Warner 1968; van

Riper et al. 1986; Asghar et al. 2015; Niebuhr et al. 2016; Paxton et al. 2016; Dadam et al. 2019). However, the infectiousness of most host species and their role in malaria transmission is poorly understood. We found a gradual relationship between parasitemia and infectiousness to mosquitoes, and enormous variation within-species in chronic parasitemia levels, which resulted in many species being capable of contributing, at least partly, to transmission. Whether other populations of *Cx. quinquefasciatus*, other species of mosquitoes, or other clades of malaria will have similarly shallow slopes between parasitemia and vector infectiousness is unknown, but this will determine the role of individual species in the transmission of this global parasite.

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Table 1. Infectiousness of Hawai‘i ‘amakihi (*Chlorodrepanis virens*) and ‘apapane (*Himatione sanguinea*) in high and low parasitemia stages of infection with *Plasmodium relictum*. Calculations of the number of infectious mosquitoes for the high and low parasitemia stage assume 10 mosquitoes feed on each host each day and are transmitting after 10 days at a temperature of 24 °C (Tables S1).

Species	High parasitemia length (days)	Mean parasite-mia: high stage	Low parasitemia length (days)	Mean parasite-mia: low stage	Mean infectious-ness: high stage	Mean infectious-ness: low stage	# infectious mosquitoes from high stage	# infectious mosquitoes from low stage	Ratio of low/high stage infectious mosquitoes
*C. virens**	42	3.6%	914	0.24%	22%	17%	92.4	1,554	16.8
H. sanguinea†	60	5.3%	1152	2.1%	23%	21%	138	2,419	17.5

*Parasitemia data from (Atkinson *et al.* 2001)

†Parasitemia data from (Yorinks & Atkinson 2000)

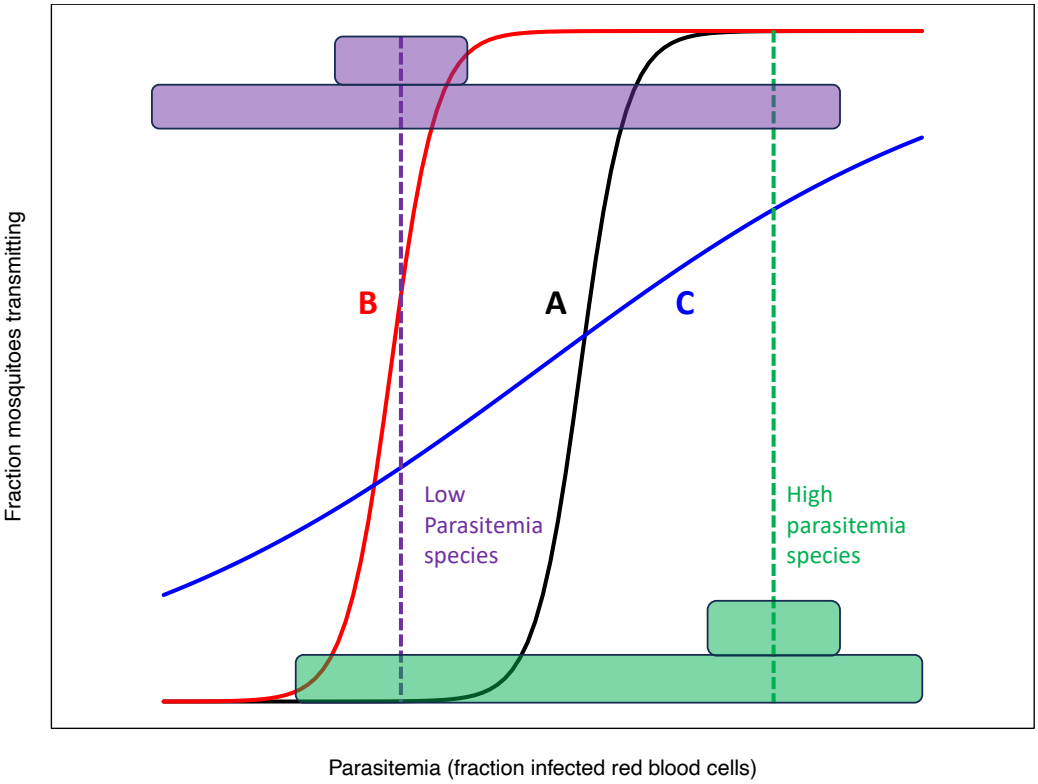


Figure 1. Possible relationships between parasitemia and infectiousness (fraction of mosquitoes that transmit malaria after feeding on that parasitemia) (lines A, B, and C), with two hypothetical species with higher and lower mean parasitemia (vertical dashed lines), and either small (short rectangles) or large (long rectangles) variation in parasitemia around the mean.

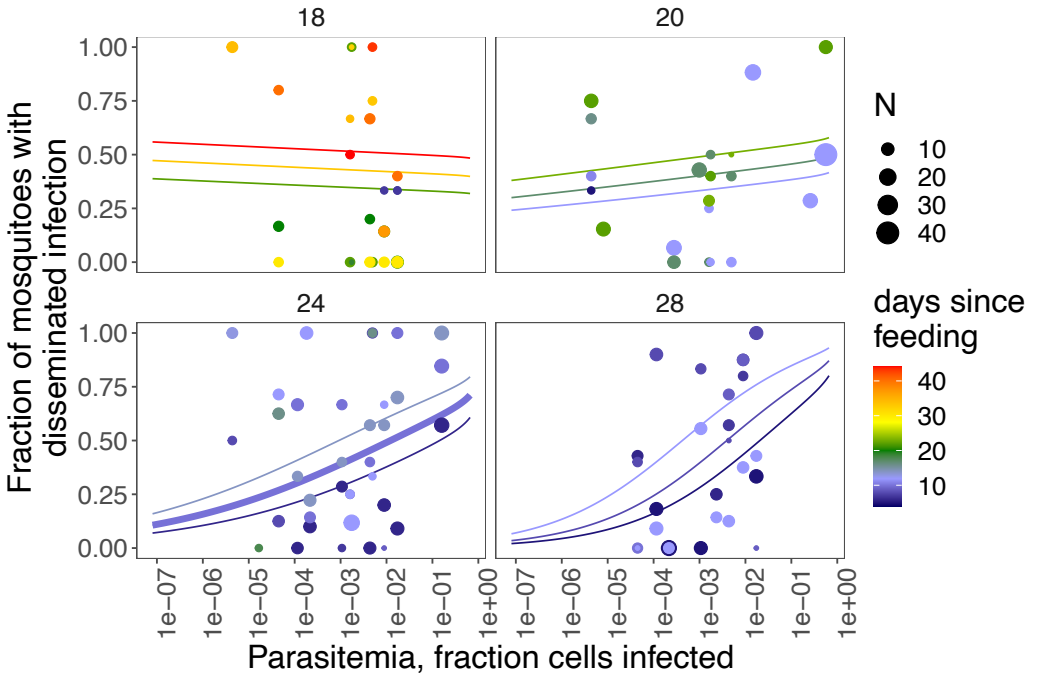


Figure 2. Fraction of mosquitoes with disseminated infections plotted against avian malaria parasitemia (fraction of red blood cells infected, on a $\log_{10}$ axis) for four temperatures (18, 20, 24, 28 °C) and a range of days since feeding. Data derived from experimental infections of domestic canaried and *Culex quinquefasciatus* mosquitoes with *Plasmodium relictum* GRW4. Points show groups of blood fed mosquitoes tested on a single day, with the size of points indicating sample size, and color showing the number of days since feeding. Colored lines show the fitted model which included bird parasitemia, days since feeding, temperature, and both two-way interactions (Table S1). The thicker line in the 24 °C panel shows the model fit for 10 days after feeding and is used for the calculations shown in Figure 3.

Figure 3. **A**) Avian malaria parasitemia (parasites per red blood cell, on a $\log_{10}$ axis) and **B**) estimated fraction of mosquitoes transmitting avian malaria 10 days after feeding on each parasitemia at 24 °C for 7 native (blue) and 10 introduced bird species (red). Points show values for individual wild-caught birds (circles) or samples from experimentally infected birds during the first 90 days post-infection taken from the literature (triangle; Atkinson et al. 1995, 2000, 2001a,b; Yorinks and Atkinson 2000). The number of wild bird samples processed is displayed above each species (total N = 743). Black circles show the mean values for each species for wild-caught birds (i.e., not including experimental infection data).

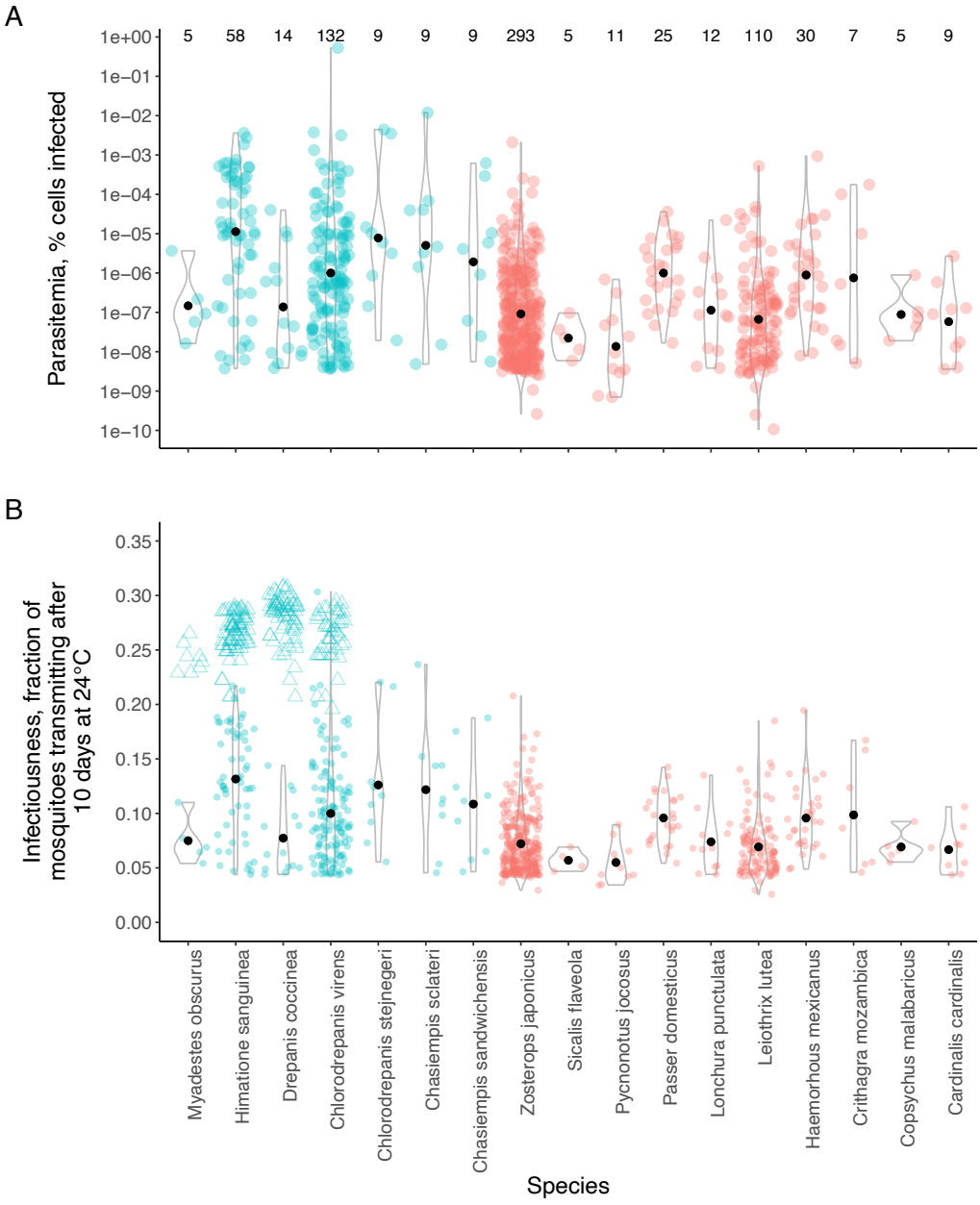
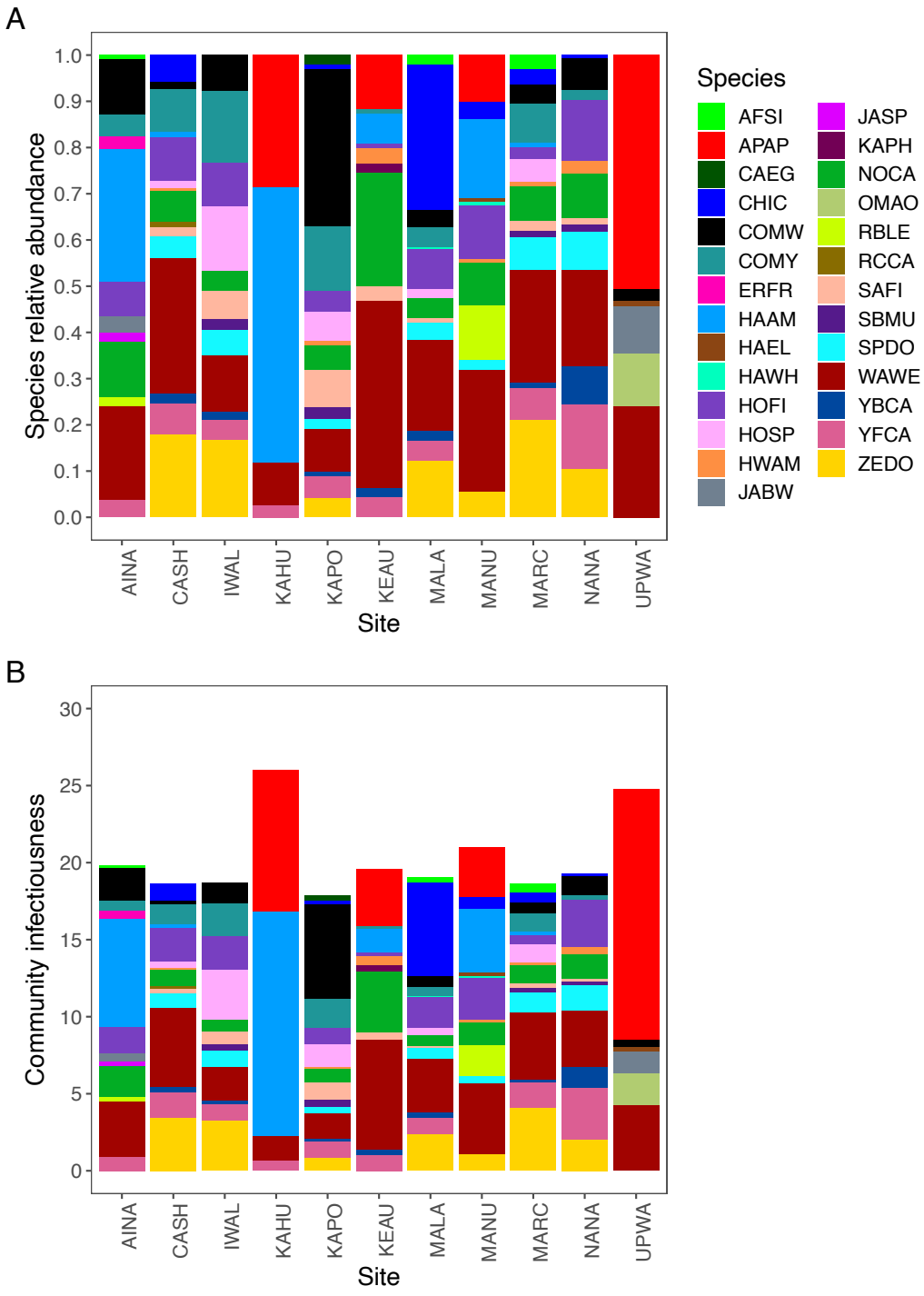

A
5
58
14
132
9
9
9
293
5
11
25
12
110
30
7
5
9
1e+00
1e−01
1e−02
1e−03
1e−04
1e−05
1e−06
1e−07
1e−08
1e−09
1e−10
Parasitemia, % cells infected
B
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Infectiousness, fraction of mosquitoes transmitting after 10 days at 24°C
Myadestes obscurus
Himatione sanguinea
Drepanis coccinea
Chlorodrepanis virens
Chlorodrepanis stejnegeri
Chasiempis sclateri
Chasiempis sandwichensis
Zosterops japonicus
Sicalis flaveola
Pycnonotus jocosus
Passer domesticus
Lonchura punctulata
Leiothrix lutea
Haemorhous mexicanus
Crithagra mozambica
Copsychus malabaricus
Cardinalis cardinalis
Species

Figure 4. Bird community composition and community infectiousness for avian malaria in Hawai‘i. **A**) Relative abundance of bird species at 11 sites based on point count sampling, with species and site acronyms listed (Tables S1, S5, S6). **B**) Contributions of species to total community infectiousness for avian malaria transmission.


A
Species relative abundance
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
AINA
CASH
IWAL
KAHU
KAPO
KEAU
MALA
MANU
MARC
NANA
UPWA
Site
Species
AFSI
APAP
CAEG
CHIC
COMW
COMY
ERFR
HAAM
HAEL
HAWH
HOFI
HOSP
HWAM
JABW
JASP
KAPH
NOCA
OMAO
RBLE
RCCA
SAFI
SBMU
SPDO
WAWE
YBCA
YFCA
ZEDO
B
Community infectiousness
30
25
20
15
10
5
0
AINA
CASH
IWAL
KAHU
KAPO
KEAU
MALA
MANU
MARC
NANA
UPWA
Site

Supplemental material

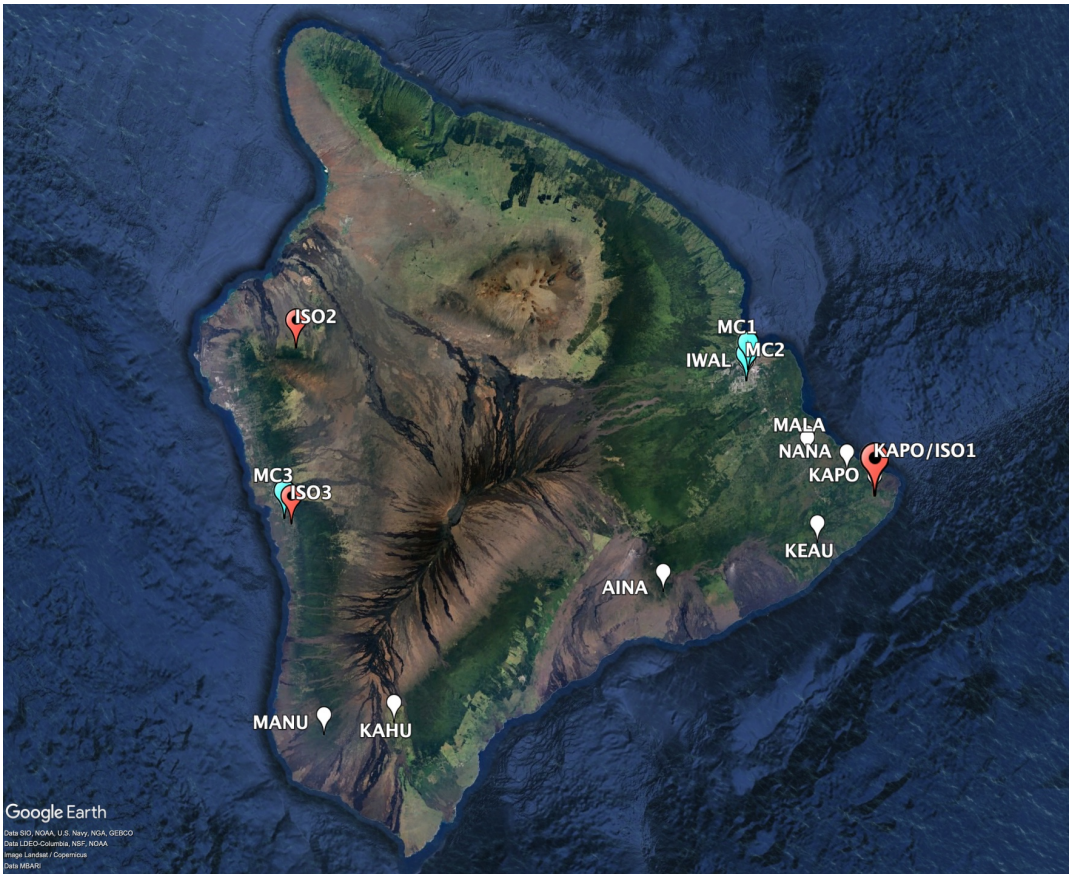


Figure S1. Collection and survey locations of study. Red points indicate the origin of *Plasmodium relictum* isolates used in experimental infection studies (ISO1, ISO2, ISO3). Blue points are collection locations for *Culex quinquefasciatus* egg rafts used for experimental infection studies (MC1, MC2, MC3). White points and one red point are point count sampling locations (described in Table S5) where we estimated bird relative abundance and community infectiousness.

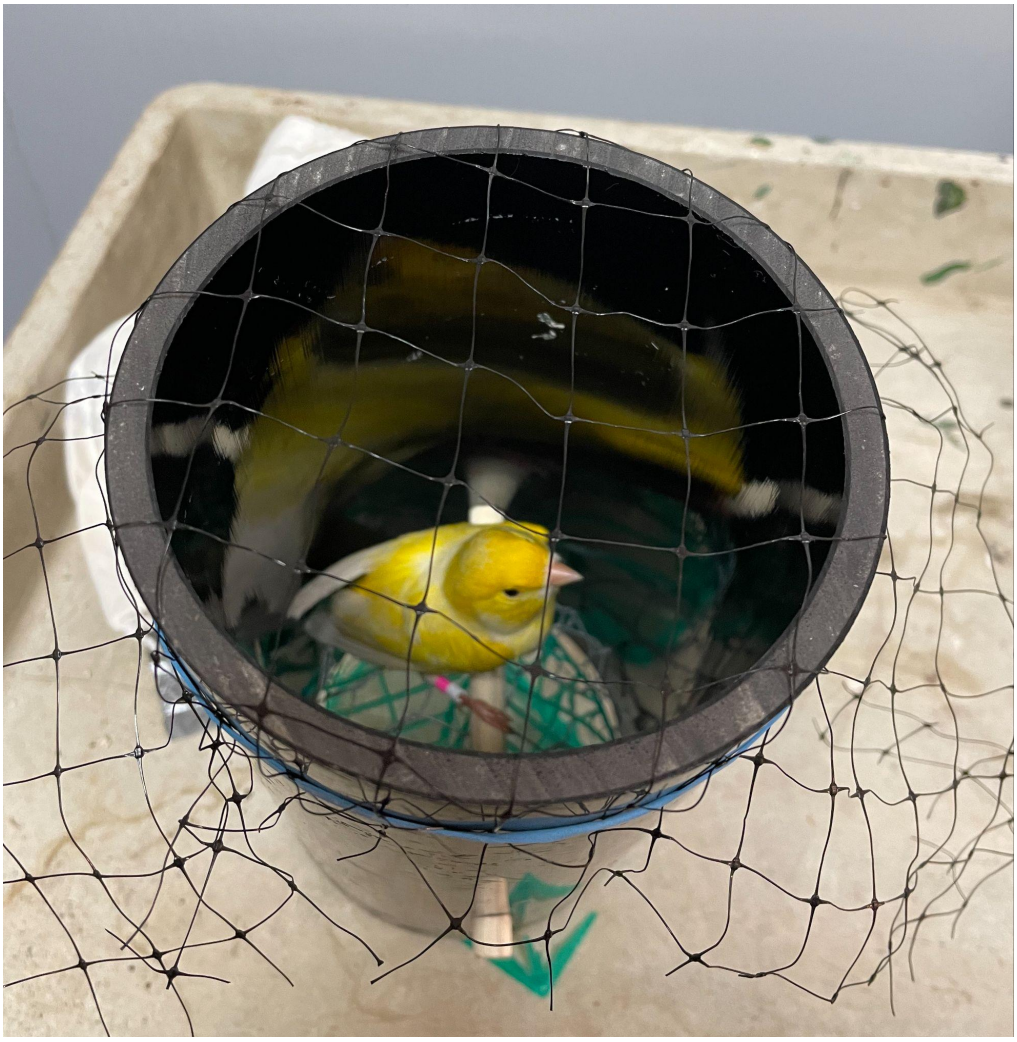


Figure S2. Mosquito feeding cylinder with an unrestrained domestic canary (*Serinus canaria*).

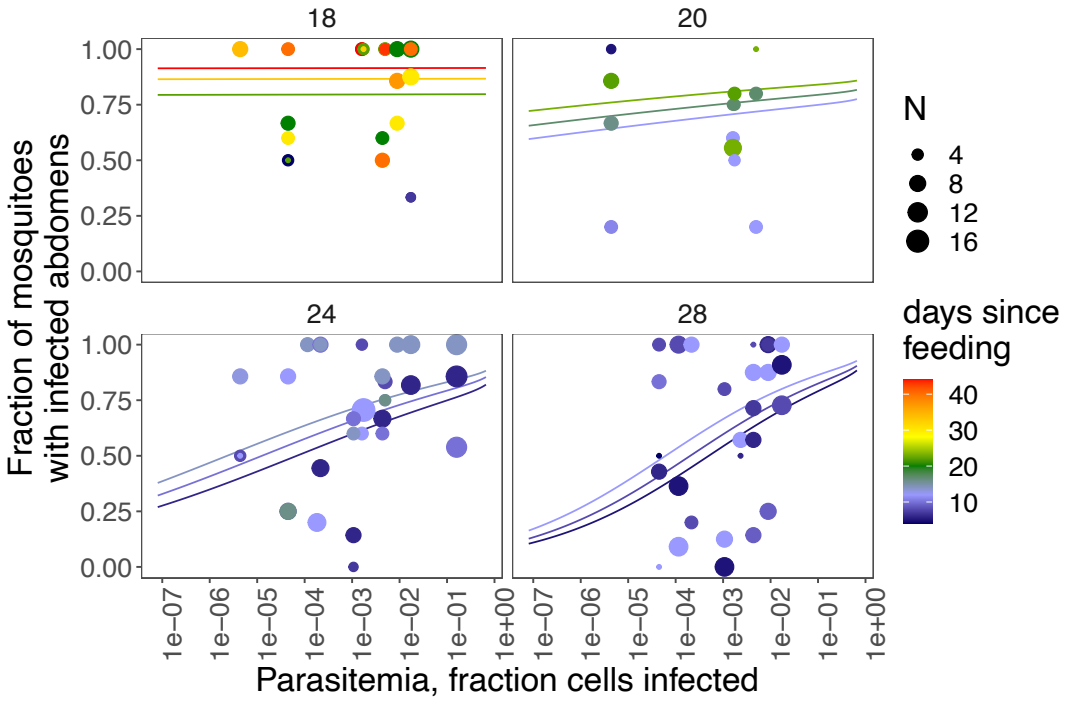


Figure S3. Fraction of mosquitoes with infected abdomens plotted against avian malaria parasitemia (fraction of red blood cells infected, on a $\log_{10}$ axis) for four temperatures (18, 20, 24, 28 °C) and a range of days since feeding. Points show groups of mosquitoes tested on a single day since feeding, with the size of points indicating sample size, and color showing the number of days since feeding. Colored lines show the fitted model which included parasitemia, days post-feeding, temperature, and both two-way interactions.

Figure S4. (A) Avian malaria parasitemia (parasites per red blood cell, on a $\log_{10}$ axis) and (B) estimated fraction of mosquitoes transmitting avian malaria 10 days after feeding on each parasitemia at 24 °C for 19 native (blue) and 22 introduced bird species (red). Points show values for individual wild-caught birds (circles) or samples from experimentally infected birds during the first 90 days post-infection taken from the literature (triangle; Atkinson *et al.* 1995, 2000, 2001a,b; Yorinks & Atkinson 2000). The number of wild bird samples that tested positive for avian malaria are displayed above each species (total N=743). Black circles show the mean values for each species for wild-caught birds (i.e., not including experimental infection data). Open circles with colored outlines are estimated values for birds without parasitemia data or low sample sizes (N<5) and are based on average parasitemia of the nearest taxonomic group.

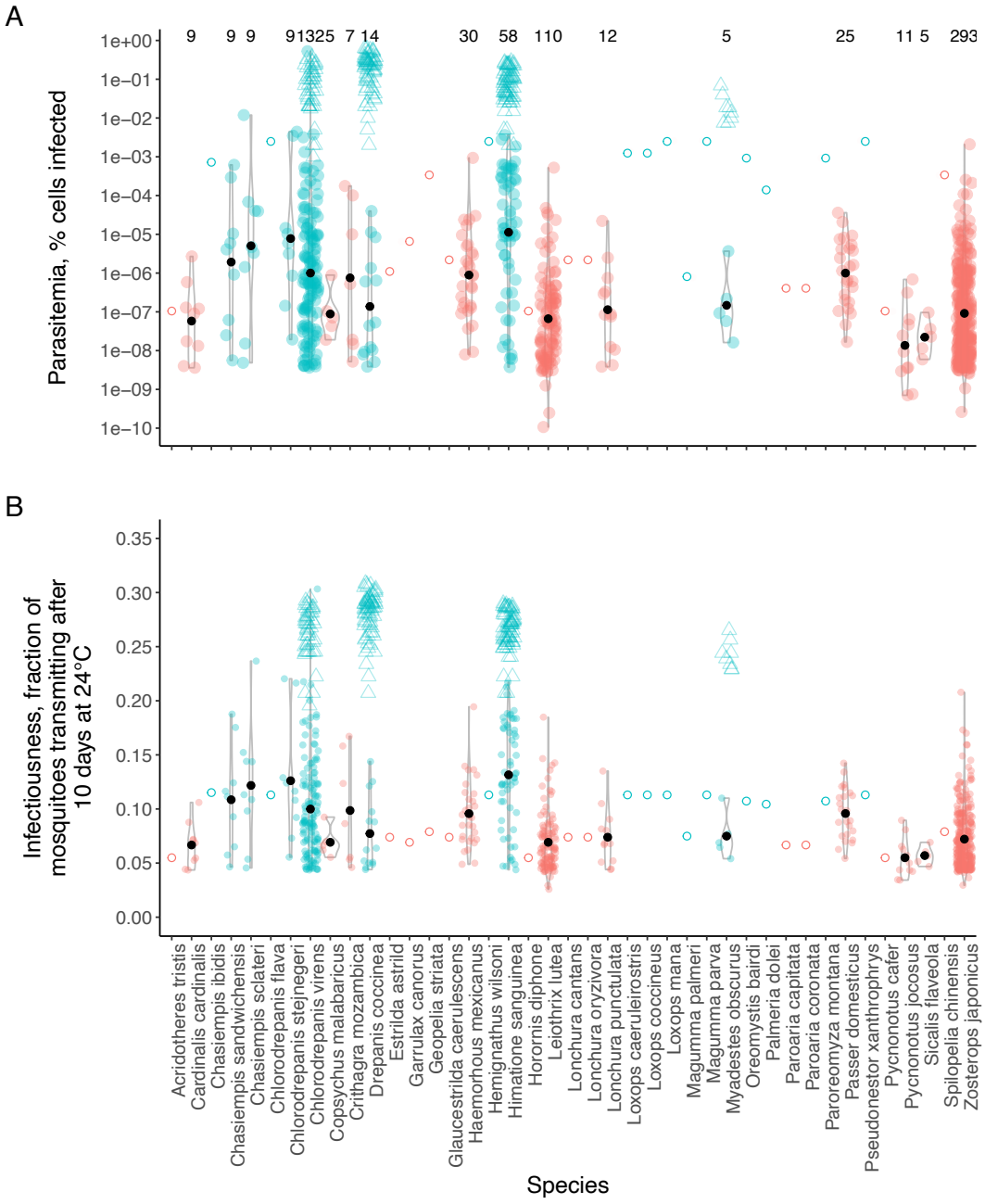
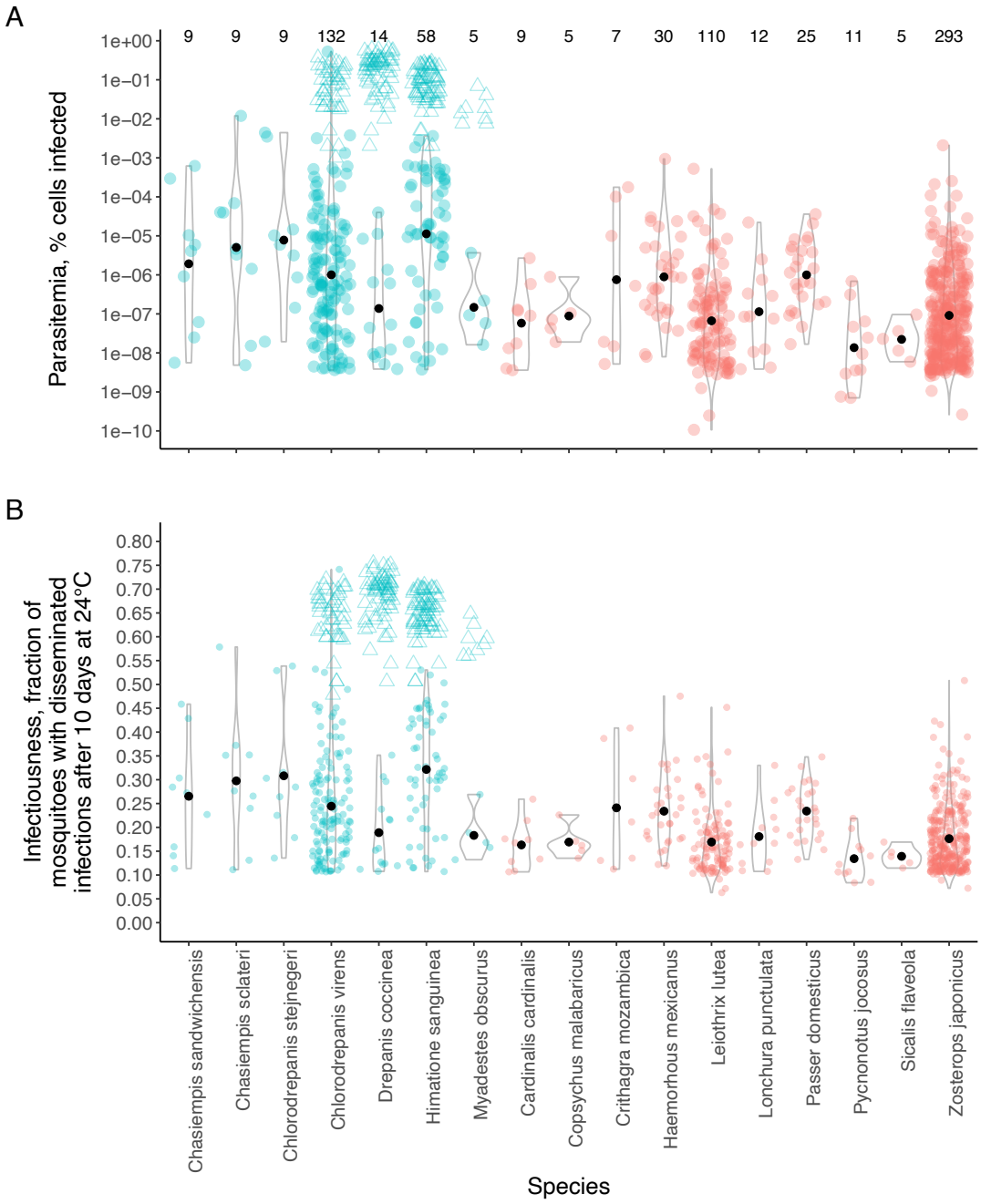

A
Parasitemia, % cells infected
1e+00
1e−01
1e−02
1e−03
1e−04
1e−05
1e−06
1e−07
1e−08
1e−09
1e−10
9
9
9
91
325
7
14
30
58
110
12
5
25
11
5
293
B
Infectiousness, fraction of mosquitoes transmitting after 10 days at 24°C
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Acridotheres tristis
Cardinalis cardinalis
Chasiempis ibidis
Chasiempis sandwichensis
Chasiempis sclateri
Chlorodrepanis flava
Chlorodrepanis stejnegeri
Chlorodrepanis virens
Copsychus malabaricus
Crithagra mozambica
Drepanis coccinea
Estrilda astrild
Garrulax canorus
Geopelia striata
Glaucestrilda caerulescens
Haemorhous mexicanus
Hemignathus wilsoni
Himatione sanguinea
Horornis diphone
Leiothrix lutea
Lonchura cantans
Lonchura oryzivora
Lonchura punctulata
Loxops caeruleirostris
Loxops coccineus
Loxops mana
Magumma palmeri
Magumma parva
Myadestes obscurus
Oreomystis bairdi
Palmeria dolei
Paroaria capitata
Paroaria coronata
Paroreomyza montana
Passer domesticus
Pseudonestor xanthrophrys
Pycnonotus cafer
Pycnonotus jocosus
Sicalis flaveola
Spilopelia chinensis
Zosterops japonicus
Species

Figure S5. **A)** Avian malaria parasitemia (parasites per red blood cell, on a $\log_{10}$ axis) and **B)** estimated mosquitoes with disseminated avian malaria infections 10 days after feeding on each parasitemia at 24 °C for 7 native (blue) and 10 introduced bird species (red). Points show values for individual wild-caught birds (circles) or samples from experimentally infected birds during the first 90 days post-infection taken from the literature (triangle; Atkinson *et al.* 1995, 2000, 2001a,b; Yorinks & Atkinson 2000). The number of wild bird samples processed is displayed above each species (total N=743). Black circles show the mean values for each species for wild-caught birds (i.e., not including experimental infection data).


A
9
9
9
132
14
58
5
9
5
7
30
110
12
25
11
5
293
Parasitemia, % cells infected
1e+00
1e−01
1e−02
1e−03
1e−04
1e−05
1e−06
1e−07
1e−08
1e−09
1e−10
B
Infectiousness, fraction of mosquitoes with disseminated infections after 10 days at 24°C
0.80
0.75
0.70
0.65
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Chasiempis sandwichensis
Chasiempis sclateri
Chlorodrepanis stejnegeri
Chlorodrepanis virens
Drepanis coccinea
Himatione sanguinea
Myadestes obscurus
Cardinalis cardinalis
Copsychus malabaricus
Crithagra mozambica
Haemorhous mexicanus
Leiothrix lutea
Lonchura punctulata
Passer domesticus
Pycnonotus jocosus
Sicalis flaveola
Zosterops japonicus
Species

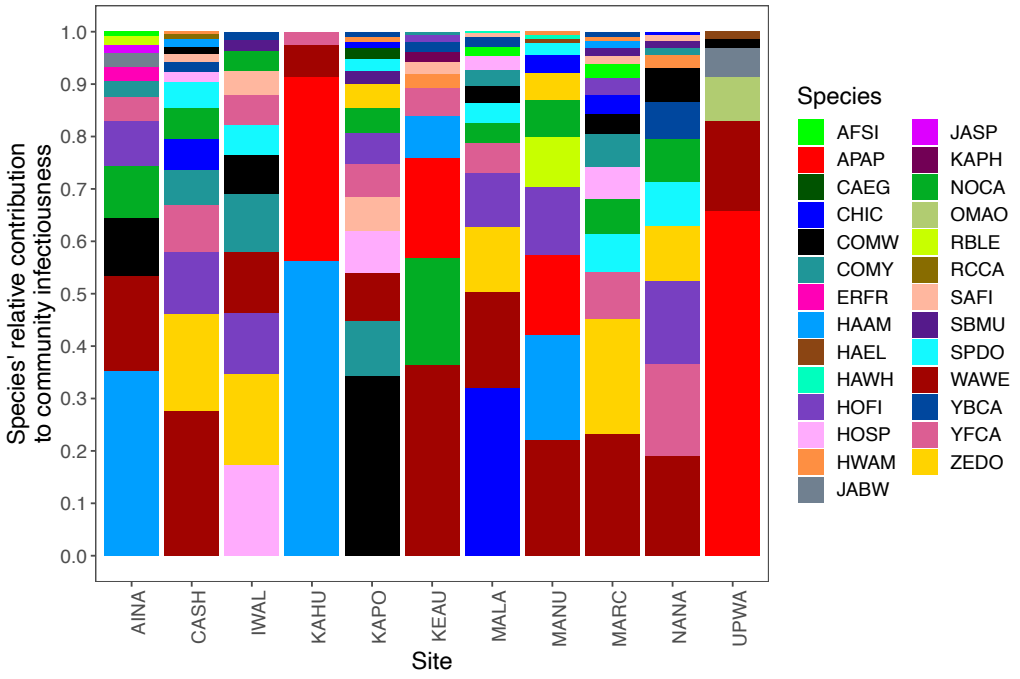


Figure S6. The proportion of avian malaria community infectiousness represented by each bird species derived from total community infectiousness values (Figure 4B, Table S6).

Table S1. Results from a generalized linear model with a binomial distribution and a logit link with the prevalence of disseminated avian malaria infections in *Culex quinquefasciatus* as the response variable and Bird Ct score, Temperature and Days since feeding and two two-way interactions as predictors.

Predictor	Coefficients	SE	z value	Pr(>|z|)
Intercept	-15.31	4.34	-3.53	0.000417
Bird Ct Score	0.514	0.152	3.83	0.000718
Temperature	0.753	0.199	3.79	0.000153
Days since feeding	-0.222	0.0755	-2.94	0.003303
Bird Ct Score*Temperature	-0.0277	0.00695	-3.99	0.0000653
Temperature*Days since feeding	0.01407	0.00398	3.54	0.000406

Table S2. Results from a generalized linear model with a binomial distribution and a logit link with the prevalence of avian malaria infections in *Culex quinquefasciatus* abdomens as the response variable and Bird Ct score, Temperature, and Days since feeding and two two-way interactions as predictors.

Predictor	Coefficients	SE	z value	Pr(>|z|)
Intercept	-9.46	6.00	-1.58	0.115
Bird Ct Score	0.373	0.20	1.87	0.0619
Temperature	0.546	0.255	2.14	0.0320
Days since feeding	-0.00400	0.0907	-0.044	0.965
Bird Ct Score*Temperature	-0.0208	0.00854	-2.44	0.0148
Temperature*Days since feeding	0.00277	0.00463	0.60	0.549

Table S3. Results from a generalized linear mixed effects model with a beta distribution and logit link with parasitemias as the response variable and a bird's status as a native or non-native (Exotic) as a fixed effect and species as a random effect (SD of random effect: 0.000071).

Predictor	Coefficients	SE	z value	Pr(>|z|)
Intercept	-6.95	0.118	-58.77	<0.00001
NativevExotic: Native	0.290	0.0776	3.73	0.000189

Table S4. Results from a generalized linear mixed effects model with a beta distribution and logit link with the prevalence of disseminated infections in *Culex quinquefasciatus* as the response variable and a bird's status as a native or non-native (Exotic) species as a fixed effect and species as a random effect (SD of random effect: 0.21).

Predictor	Coefficients	SE	z value	Pr(>|z|)
Intercept	-1.54	0.0736	-20.9	<0.00001
NativevExotic: Native	0.389	0.112	3.46	0.000534

Table S5. Point count survey locations for Hawai‘i Island in 2019-2020. Latitude/Longitude location is the center of a 4-hectare (200 m x 200 m) area. Species relative abundances used in Figure 3 are the sum of the total observations of a species divided by the total of all species observed over four sequential six-minute surveys conducted 200 m apart.

Site Name	Site Code	Forest Description	Latitude	Longitude	Elevation (m)	Survey Date
Iwalani Hilo, HI	IWAL	Suburban, city	19.6858	-155.0778	29	4/23/20
Hawai‘i Paradise Park Site 1, HI	MARC	Agricultural	19.5777	-154.9659	60	5/9/19
Kepoho, HI	KEPO	Agricultural	19.4985	-154.8537	62	4/16/20
Hawai‘i Paradise Park Site 2, HI	CASH	Agricultural	19.5682	-154.9724	77	5/9/19
Nanawale Forest Reserve, HI	NANA	Wet ohia forest	19.5373	-154.9032	100	3/16/20
Malamalama Waldorf School, HI	MALA	Agricultural	19.5586	-154.9728	105	5/9/19
Keauohana Forest Reserve, HI	KEAU	Wet ohia forest	19.4188	-154.9524	250	4/15/20
Manuka State Park, HI	MANU	Dry ohia forest	19.1105	-155.8246	600	5/11/19
Ainahou Ranch Unit, Hawai‘i Volcanoes National Park, HI	AINA	Wet ohia forest	19.3436	-155.2305	921	3/11/20
Upper Waiakea Forest Reserve, HI	UPWA	Wet ohia forest	19.5672	-155.2306	1100	1/30/20
Kahuku Ranch Unit, Hawai‘i Volcanoes National Park, HI	KAHU	Dry ohia forest	19.1336	-155.7005	1270	5/23/19

Table S6. Species observed in point count surveys from locations on Hawai‘i Island in 2019-2020. Observed at sites in Table S5, Figure 4.

Species Code	Common Name	Genus	Species	Family
AFSI	African silverbill	*Lonchura*	*cantans*	Estrildidae
APAP*	‘Apapane	*Himatione*	*sanguinea*	Fringillidae
CAEG	Cattle egret	*Bubulcus*	*ibis*	Ardeidae
CHIC	Domestic chicken	*Gallus*	*gallus*	Phasianidae
COMY*	Common myna	*Acridotheres*	*tristis*	Sturnidae
COMW*	Common waxbill	*Estrilda*	*astrild*	Estrildidae
ERFR	Erckel's francolin	*Pternistis*	*erckelii*	Phasianidae
HAAM*	Hawai‘i ‘amakihi	*Chlorodrepanis*	*virens*	Fringillidae
HAEL*	Hawai‘i ‘elepaio	*Chasiempis*	*sandwichensis*	Monarchidae
HAWH	Io (Hawaiian hawk)	*Buteo*	*solitarius*	Accipitridae
HOFI*	House finch	*Haemorhous*	*mexicanus*	Fringillidae
HOSP*	House sparrow	*Passer*	*domesticus*	Passeridae
HWAM	Chinese hwamei	*Garrulax*	*canorus*	Leiothrichidae
JABW*	Japanese bush warbler	*Horornis*	*diphone*	Cettiidae
JASP	Java sparrow	*Lonchura*	*oryzivora*	Estrildidae
KAPH	Kalij pheasant	*Lophura*	*leucomelanos*	Phasianidae
NOCA*	Northern cardinal	*Cardinalis*	*cardinalis*	Cardinalidae
OMAO	‘Ōma‘o	*Myadestes*	*obscurus*	Turdidae
RBLE*	Red-billed leothrix	*Leiothrix*	*lutea*	Leiothrichidae
RCCA*	Red-crested cardinal	*Paroaria*	*coronata*	Thraupidae
SAFI*	Saffron finch	*Sicalis*	*flaveola*	Thraupidae
SBMU*	Scaly-breasted munia	*Lonchura*	*punctulata*	Estrildidae
SPDO	Spotted dove	*Spilopelia*	*chinensis*	Columbidae
WAWE*	Warbling white-eye	*Zosterops*	*japonicus*	Zosteropidae
YBCA*	Yellow-billed cardinal	*Paroaria*	*capitata*	Thraupidae
YFCA*	Yellow-fronted canary	*Crithagra*	*mozambica*	Fringillidae
ZEDO	Zebra dove	*Geopelia*	*striata*	Columbidae

**P. relictum* GRW4 infection known for this species based on this study or experimental infection studies.

CONCLUSION

Multi-host pathogens can pose a serious threat to human health, economy, and wildlife conservation (Cleaveland *et al.* 2001). The majority of human and livestock pathogens are multi-host (Cleaveland *et al.* 2001), and several multi-host species severely impact wildlife populations (Cheng *et al.* 2011; Frick *et al.* 2010; van Riper *et al.* 1986). Host community composition and environmental conditions are two ecological factors that determine the transmission of multi-host pathogens (Harvell *et al.* 2002; LoGiudice *et al.* 2003; McClure *et al.* 2020). Anthropogenic activities alter both host communities and environmental conditions with consequences that often complicate accurate predictions of transmission and our ability to manage disease risk (Kilpatrick *et al.* 2008; LoGiudice *et al.* 2003; Paull *et al.* 2017; Rohr *et al.* 2011). In Hawai‘i, a multi-host avian malaria species (*P. relictum*) provides an ideal system to closely examine how host community and environmental conditions affect transmission. Avian malaria is a major conservation concern because it negatively affects native Hawaiian bird populations and threatens species with extinction (Paxton *et al.* 2022; van Riper *et al.* 1986; Samuel *et al.* 2011). The reduction of avian malaria transmission is a top priority, and an improved understanding of avian malaria ecology will help guide conservation management (Haleakalā National Park 2022).

My dissertation research suggests many species and possibly most host communities in Hawai‘i support avian malaria transmission. A primary aim of this

work was to quantify the relationship between bird parasitemia and the infectiousness of biting mosquitoes to determine which birds contribute to avian malaria transmission. Addressing this aim required a combination of laboratory experimental infection studies that measured the fraction of infectious mosquitoes produced from feedings on different bird parasitemias and extensive parasitemia data from wild bird species in Hawai‘i. My experimental infection studies uncovered a gradual relationship between host parasitemia and mosquito infectiousness, resulting in many parasitemias creating infectious *Cx. quinquefasciatus* mosquitoes, including extremely low parasitemias. The wild bird parasitemia data documented infection in many species and large within-species variation in parasitemias. I then used the relationship between microscopy of blood smears and qPCR that I developed in my second chapter to convert field, lab, and literature parasitemia data into a common quantitative metric that could be translated into mosquito infectiousness. Translating wild bird parasitemias into mosquito infectiousness revealed considerable overlap in the infectiousness of native and non-native bird species. Thus, the combination of the laboratory and wild bird sampling data described in this dissertation indicates many species of birds could play an important role in infecting mosquitoes with avian malaria. Furthermore, using the same method, I was able to compare the number of mosquitoes produced by the short high parasitemia acute stage of avian malaria infection with the longer low parasitemia chronic stage. Infected birds spend the majority of their lives in the chronic stage and these individuals comprise the majority of infected birds within a community (Atkinson *et al.* 2001; Atkinson & Samuel

2010; Kilpatrick *et al.* 2006b). I found that the low parasitemia chronic stage of infection produced many more infectious mosquitoes than the high parasitemia acute stage across the lifespans of two Hawaiian bird species. This suggests that for some species, chronic infections are the most important stage for avian malaria transmission in Hawai‘i. Altogether, because many bird species are infectious to mosquitoes in both their acute and chronic infection stages, most bird communities in Hawai‘i transmit avian malaria.

My dissertation also highlights how warming global temperatures will increase avian malaria transmission in Hawai‘i. I found the relationships between temperature and key Hawaiian *Cx. quinquefasciatus* life history traits indicate mosquito populations have already increased in Hawai‘i over the last 10 years and are likely to continue increasing with 2 ℃ of warming. Larger mosquito populations increase the number of contacts between avian malaria hosts and mosquitoes, which increases the probability of transmission. However, transmission is modulated by mosquito feeding preferences and thus host community composition (McClure *et al.* 2020). My finding that the slope of the relationship between bird parasitemia and mosquito infectiousness increases with temperature suggests warming will increase the number of infectious mosquitoes produced by high parasitemia birds. Native birds have higher average parasitemias than non-native birds, and their density within communities is positively correlated with the fraction of infected mosquitoes (McClure *et al.* 2020). Consequently, warming temperatures could increase the infectiousness of native birds and increase avian malaria transmission if mosquitoes

preferentially feed on them. The synergy of these effects could exacerbate the disease-mediated declines of the most susceptible species in warming habitats where numerous native bird species occur. Not only are warming temperatures increasing transmission in these habitats by increasing mosquito populations but also by increasing the infectiousness of the host community.

More generally, my dissertation improves our understanding of the conditions that have allowed *Plasmodium relictum* to become a globally distributed multi-host pathogen and a significant conservation concern in Hawai‘i. First, *P. relictum* appears to be well adapted to infecting a large number of host species, including many of the native and introduced species I sampled in Hawai‘i. It also has the ability to chronically infect many of these hosts, evading complete eradication by their immune responses (Atkinson & Samuel 2010; Jarvi *et al.* 2002; Kilpatrick *et al.* 2006b; Knowles *et al.* 2010). Many avian haemosporian parasites have evolved a chronic infection life history strategy (Allan & Mahrt 1989). They can persist at low levels in the bloodstream or produce dormant infection stages in other host cells that relapse into the bloodstream in response to triggers such as mosquito bites (Cornet *et al.* 2014). My experimental infection work provides substantial evidence for the success of this life strategy. I found that many of the low parasitemias present in chronically infected birds in Hawai‘i create infectious mosquitoes. The ability of *P. relictum* to transmit from chronically infected hosts and respond to vector cues facilitates its distribution in both temperate regions with seasonal mosquito availability and tropical regions, such as Hawai‘i, where mosquitoes are present year-round in many locations

(Cornet *et al.* 2014; Pigeault *et al.* 2018; van Riper *et al.* 1986). Second, *P. relictum* has adapted to infect a globally distributed and highly competent mosquito vector (*Cx. quinquefasciatus*). *Culex* mosquitoes are frequently ornithophilic, or bird-biting, which increases the likelihood of *P. relictum* transmission to a susceptible bird (Farajollahi *et al.* 2011; Kilpatrick *et al.* 2006a; Savage *et al.* 2007). However, *Culex* populations vary in their susceptibility to *P. relictum* infection (Huff 1934). Our records of extremely low bird parasitemias creating infectious mosquitoes, the lowest yet recorded for *P. relictum*, agree with other observations (LaPointe *et al.* 2005, 2010) that Hawaiian *Cx. quinquefasciatus* are highly competent vectors. Small changes in mosquito susceptibility to *P. relictum* infection could have some of the most significant effects on avian malaria transmission. For example, if Hawaiian *Cx. quinquefasciatus* populations evolve to resist infection from low parasitemias, birds with parasitemias below the new parasitemia threshold will become dead-end hosts. Avian malaria transmission could be significantly reduced in communities with many low parasitemia birds, with the extent of the reduction dependent on the feeding preferences of Hawaiian *Cx. quinquefasciatus* (Kilpatrick *et al.* 2006a; McClure *et al.* 2020). Finally, suitable thermal conditions for *P. relictum* and *Cx. quinquefasciatus* development are widespread and increasing with climate change. The global prevalence of avian malaria appears to be increasing in parallel with climate change (Garamszegi 2011). The mechanisms by which temperature affects mosquito population sizes and distribution, and thus contact rates with hosts, have been well described here and in other works (Ciota *et al.* 2014; Mordecai *et al.* 2019; Moser *et*

al. 2023; Rueda *et al.* 1990), so too has the relationship between temperature and the rate of *Plasmodium* development within mosquitoes (LaPointe *et al.* 2010). However, the novel finding that warming temperatures increase the infectiousness of many host parasitemias may help explain the increase in avian malaria prevalence at both the thermal margin of malaria transmission (e.g., high-elevation habitats) and within the core.

Taken together, this work underscores the importance of using multiple approaches to reduce avian malaria transmission and aid the recovery of native bird populations. First, vector-control strategies should focus on reducing contact rates between birds and mosquitoes, especially in habitats with numerous high parasitemia native birds and thermally suitable conditions for avian malaria transmission. This could take the form of an incompatible insect technique, which uses a maternally inherited endosymbiotic bacteria *Wolbachia* to suppress mosquito reproduction (Laven 1967), or reduction of mosquito larvae and larval habitats. For example, *Bacillus thuringiensis israelensis* (Bti), an EPA-registered environmentally-safe, effective, and target-specific biocide, could be applied to kill mosquito larvae (Stoytcheva 2011). Alternatively, transmission could be suppressed by reducing the competence of *Cx. quinquefasciatu*s mosquitoes for avian malaria. This would require the replacement of existing *Cx. quinquefasciatu*s populations in Hawai‘i with more refractory individuals derived either from selective breeding, genetic modifications, or with *Wolbachia* infections that interfere with avian malaria transmission (Nazni *et al.* 2019; Pascini *et al.* 2022; Ross *et al.* 2022). Mosquito control should also be

paired with control of introduced predators (e.g., cats, rats, mongoose) at low and middle elevations. This will increase the survival of native birds and help facilitate the continued evolution of resistance or tolerance to avian malaria that is already developing in some native species (Atkinson *et al.* 2013; Atkinson & Samuel 2010; Kilpatrick 2006; Woodworth *et al.* 2005).

My thesis advances our understanding of avian malaria transmission and ecology and highlights key future research needs. Additional work on vector feeding preferences is needed to clarify the role of different avian species in transmission. The parasitemias of the bird species mosquitoes prefer to feed upon will determine the intensity of transmission within communities. My work provides critical data to calculate how feeding patterns and community composition determine avian malaria transmission. Future work should also continue to examine both avian malaria parasitemia and prevalence data to further elucidate which mechanisms or combinations of mechanisms cause changes in avian malaria transmission. Such research should critically include genomic approaches to detect potential changes in genes associated with host/vector resistance or pathogen virulence. Exploring the genetic components of host-pathogen-vector interactions is important to a holistic understanding of avian malaria transmission now and as the system evolves into the future.

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