‘Amakihi kaulana i ka hoʻōla maʻi: Assessing the long-term impacts of chronic infection with avian malaria in Hawaiʻi ʻamakihi

A Thesis submitted to the Graduate Division of the
University of Hawaiʻi at Hilo in partial fulfillment
of the requirements for the degree of

Master of Science
In
Tropical Conservation Biology and Environmental Science

May 2024

By Emma Stierhoff

Thesis Committee:
Patrick Hart, Chairperson
Susan Jarvi
Kristina Paxton
Eben Paxton

Keywords: Avian malaria, Hawaiʻi ʻamakihi, chronic infection, ecological physiology, blood analysis, oxidative stress, hematocrit, triglycerides, body condition

ACKNOWLEDGEMENTS

This project was made possible by an amazing group of collaborators and supporters that I am so appreciative to have worked with. Mahalo to my advisor Patrick Hart, who was the first person I connected with at University of Hawaiʻi (UH), the reason I came here, and who has been so incredibly supportive throughout my academic career. Mahalo to my committee members and main collaborators, Eben Paxton and Kristina Paxton, for allowing me to join such a meaningful and informative project, and for all their support throughout the process. This project would not have been possible without them. I have learned so much from working with them, and am grateful to have helped get the ʻĀinahou project running with them. Additionally, mahalo to Susan Jarvi, whose past work on avian malaria helped set the foundation for this project, and who was a very supportive committee member.

My thesis would not be what it is without the invaluable assistance from all the United States Geological Survey (USGS) volunteers and interns that contributed: Carleigh Stein, Maëliss Hoarau, Wade Burris, Liliana Tobar, and Makoa De Almeida. This project is as much theirs as it is mine. I am immensely grateful for all the time they devoted to collecting, processing, and analyzing samples throughout this project. They are amazing researchers and people, and it was a pleasure to get to know them through this work.

I am also incredibly grateful to the many other folks who have come help band and collect blood samples throughout this project including Josie Tupu, Braxton Igne, Paulo Ditzel, Lauren Smith, Noah Hunt, Meeya Odell, and everyone else who came out banding with us. Also to Ann Tanimoto Johnson, for her endless support and for always providing whatever I needed for this project.

To the organizations that helped make this possible by providing resources and making this fieldwork possible, particularly Hawaiʻi Volcanoes National Park and USGS, I am so very grateful. Folks at USGS in particular provided invaluable support for this project, allowing me to use their lab facilities and supporting the ongoing banding efforts for this project. Mahalo especially to Renee Bellinger, Corinna Pinzari, Kristi Fukunaga, and Yasemin Can, for all their work developing the lab protocols for this project and carrying out analyses.

Mahalo nui to American Ornithological Society (AOS) for providing additional funding for this project. Additionally, mahalo to Xiuxiu Sun and Zahra Yusufali with United States Department of Agriculture (USDA) for their immense generosity in allowing me to use their facility for my lab analyses and all their support throughout that process. Lastly, mahalo to UH Hilo for being the home base from which I could complete this work, and for supporting my graduate education through a graduate assistantship.

I would not be where I am today without the friends and family who have supported me unequivocally. Mahalo to my parents, Valerie and Paul Stierhoff for prioritizing my education, for supporting me even when I was called to move across the world, and for encouraging me the whole way through. To the friends I‘ve made here, and the the ones who have stuck by my side from throusands of miles away, mahalo for supporting me through some of the most turbulent, but also happiest and most growth-filled years of my life. Their support has kept me going.

Mahalo nui to the ‘Āina of Hawai‘i for nourishing me physically, emotionally, and intellectually throughout my time here. I have felt so much abundance in my life these past few years, and it has helped me grow immensely. Lastly, I would be remis if I did not specifically thank the ‘amakihi. As much as I try to claim I‘m not a bird person, the goofy grin on my face every time I see an ‘amakihi gives me away. It has been one of the greatest joys and privileges of my life to work with these goofy, gentle, sassy, resilient manu. Their strength gives me hope for the future of Hawaiian forest birds. They are the greatest contributors to this research, and the foundation for this project. I can only hope to give back as much as they have given me. Hopefully this thesis is a start.

ABSTRACT

The introduction of avian malaria (*Plasmodium relictum*) to Hawai‘i has decimated native forest bird populations, driving many species to extinction, and threatening those that remain. However, one native honeycreeper, the Hawai‘i ‘amakihi (*Chlorodrepanis virens*, hereafter referred to as ‘amakihi), has shown resilience against acute infection with avian malaria. ‘Amakihi who survive the acute stage remain chronically infected with low parasitemia levels. Although immediate costs of acute malarial infection have been closely studied, the long-term costs of chronic infection are poorly understood in this species. I assessed the impact of chronic infection on the physiological condition of ‘amakihi near ‘Āinahou Ranch in Hawai‘i Volcanoes National Park. During biweekly banding sessions from May 2022 to August 2023, ‘amakihi were banded, measured for tarsus and wing chord, and weighed, and a blood sample was collected for each bird. Using the blood samples, I measured hematocrit, triglycerides, and reactive oxygen metabolites (ROMs) to assess physiological condition, and I determined disease status (undetermined or positive) using qPCR. There were no detectable differences in hematocrit, triglycerides, nor ROMs based on disease status. Body condition was slightly higher in birds that could be confirmed chronically infected than those that were undetermined, but this difference did not persist when accounting for all infected birds. Although acute infection with avian malaria has been shown to have significant costs, this study suggests that ‘amakihi surviving acute infection may not have negative long-term fitness impacts. These results further demonstrate the resilience ‘amakihi have against avian malaria and set the foundation for future research to determine factors facilitating this resilience and how that knowledge might be applied to protect more threatened honeycreeper populations.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS……………………………………………………………………….i
ABSTRACT……………………………………………………………………………ii
LIST OF FIGURES…………………………………………………………………….v
INTRODUCTION…………………………………………………………………...1
METHODS……..………………………………………………………………6
Study Site ……………………………………………………………….……6
Sample Collection ……………………………………………………………..8
Classifying life stage ………………………………………………………......9
Genetic Sexing…………………………………………………………………...10
Determining disease status ……………………………………………………...10
Oxidative Stress ………………………………………………………………12
Triglycerides …………………………………………………………………….13
Statistical Analysis ……………………………………………………………13
Permitting …………………………………………………………………….17
RESULTS……………………………………………………………………………..17
Infection Rate ………………………………………………………………17
Hematocrit ……………………………………………………………………18
Oxidative Stress ………………………………………………………………18
Triglycerides …………………………………………………………………….19
Body Condition ……………………………………………………………….19
DISCUSSION……………………………………………………………………20
Hematocrit ……………………………………………………………………21
Oxidative Stress ………………………………………………………………21
Triglycerides …………………………………………………………………….22
Body Condition ……………………………………………………………….22
C_T *value and physiology* ……………………………………………………...23
Limitations ……………………………………………………………………23
CONCLUSIONS…………………………………………………………………...24
APPENDIX…………………………………………………………………….…...26
LITERATURE CITED ……………………………………………………………….30

LIST OF FIGURES

Figure 1. Hematocrit levels based on disease status in ‘amakihi……………………………………………………………………………………26

Figure 2. Hematocrit levels plotted against square root transformed Cq value……………………………………………………………………….26

Figure 3. Square root transformed ROMs plotted against log transformed triglycerides……………………………………………………………….27

Figure 4. Square root transformed ROMs levels based on disease status in ‘amakihi……………………………………………………………….…...27

Figure 5. Square root transformed ROMs levels plotted against square root transformed Cq value………………………………………………………..27

Figure 6. Log transformed triglyceride levels based on time of day blood sample was taken…………………………………………………………..28

Figure 7. Log transformed triglyceride levels based on disease status in ‘amakihi……………………………………………………………….…...28

Figure 8. Log transformed triglyceride levels plotted against square root transformed Cq value………………………………………………………..28

Figure 9. Body condition by disease status in all infected ‘amakihi………………………………………………………………………………….29

Figure 10. Body condition plotted against square root transformed Cq value……………………………………………………………………….29

Figure 11. Body condition by disease status in ‘amakihi when only including positive ‘amakihi confirmed to have chonic malarial infection……………………………………………………………29

E ia aʻe ka ʻAmakihi, manu kupaianaha o uka	*Behold the ʻAmakihi, supernatural bird of the uplands*
Manu holu napenape,	*Resilient, he who springs back*
ʻAmakihi kaulana i ka hoʻōla maʻi	*Famous ʻAmakihi who overcomes disease*
ʻIke ʻia ma ke kulanui o Mānoa	*Visible at the college of Mānoa*
A i Pelekunu ma Molokaʻi	*All the way to Pelekunu on Molokaʻi*
Mai Kalāpana a i Makuʻu ma Puna	*From Kalapana down to Makuʻu in Puna*

Section of *O ka lele a nei auna*, an Oli written by Kekuhi Kealiʻikanakaʻoleohaililani and ʻAhuimanu

INTRODUCTION

Hawaiʻi is home to a large variety of endemic forest birds. Perhaps most well-known are the Hawaiian honeycreepers. About 5.5 million years ago, rosefinches (family *Fringilidae*) blown off course landed in Kauaʻi, and over time moved across Hawaiʻi and rapidly evolved into 55 different species and subspecies of honeycreepers. These birds have historically thrived on the Hawaiian archipelago, and are significant to kānaka maoli culture, serving both utilitarian and spiritual roles (Amante-Helweg & Conant 2009). However, following European contact, habitat loss and introduced species led to widespread population declines among native birds. Concerns of such circumstances were originally voiced by kānaka maoli (native Hawaiians), such as Penukahi, who warned, "The ʻiʻiwi, the oʻu, the ʻakakane, the ʻamakihi, the oʻolomao, the elepaio, these are the natives that are lost… because the bad birds are increasing among us and they harm the plants, and the wild food plants" (Amante-Helweg & Conant 2009). However, such concerns were ignored by settlers until the mid-twentieth century, at which point many endemic species reached extinction, and the remainder were largely threatened. Of the 55 honeycreeper species and subspecies that evolved, only 17 of these species remain. One of the major threats faced by native Hawaiian honeycreepers is introduced diseases such as avian malaria.

Avian malaria is a deadly disease caused by a protozoan parasite (*Plasmodium relictum*) that afflicts wild birds across the world. At the acute stage of infection when parasitemia is highest, birds frequently experience weight loss, anorexia, enlargement and discoloration of the liver and spleen, and anemia (Atkinson et al. 2000; LaPointe et al. 2012). Since its introduction to Hawaiʻi in the early twentieth century, avian malaria has devastated native bird populations and caused multiple extinctions (van Riper III et al. 1986). The vector for avian malaria in Hawaiʻi, the southern house mosquito (*Culex quinquefasciatus*), mostly occurs at low elevations (<1,500 m) because it requires warm temperatures to develop (LaPointe et al. 2010). As a result, most native forest birds have been driven to higher elevations that lack *Culex* mosquitos. However, *Culex* mosquitos are predicted to move up in elevation due to climate change and associated rising temperatures, which will result in the loss of disease-free, high-altitude refugia (Liao et al. 2015). Without immediate intervention, many more extinctions are predicted to result from avian malaria across Hawaiʻi within the next few decades (Paxton et al. 2016a). Thus, finding ways to mitigate the impact of avian malaria on native birds is a pressing conservation concern.

Some populations of Hawai‘i ‘amakihi (*Chlorodrepanis virens*, hereafter referred to as ‘amakihi), a native Hawaiian honeycreeper, may be more resilient to infection with avian malaria than other native birds. Recorded mortality rates from acute malarial infection in ‘amakihi range from as low as 2.5% in a low-elevation population to as high as 65% in a high-elevation population (Atkinson et al. 2000; Samuel et al. 2015). Their mortality rates are generally lower than those observed in other native honeycreepers like ‘apapane (*Himatione sanguinea*) and ‘i‘iwi (*Drepanis coccinea*) whose mortality rates are 63% and 90% respectively (Atkinson et al. 1995; Yorinks & Atkinson 2000). Their resistance to avian malaria is likely what allows ‘amakihi to persist at lower elevations despite the high prevalence of avian malaria. However, like the other honeycreepers, acutely infected ‘amakihi still suffer anorexia, weight loss, and lethargy, particularly at peak parasitemia (Atkinson et al. 2000). Although they can survive in areas with avian malaria, ‘amakihi are still heavily impacted by the disease.

The effects of avian malaria on Hawai‘i ‘amakihi vary widely across different populations. Variation in response to acute infection with avian malaria, particularly the broad range of mortality rates, may be owed to genetic differences across populations (Eggert et al. 2008). It is likely that low-elevation ‘amakihi populations were founded by birds with genes that facilitated resilience against avian malaria and thus, they survived the introduction of the pathogens in the 1900s (Foster et al. 2007; Eggert et al. 2008). ‘Amakihi at mid-elevations appear to be most at-risk because they do not have as much resilience towards avian malaria as those at low elevations, and they are exposed to an intermediate malarial infection rate (Samuel et al. 2015). Because the impact of avian malaria on ‘amakihi is so variable by population, population-specific studies are necessary to fully understand the costs of this disease.

Previous research suggests that ‘amakihi who survive acute infection with avian malaria remain chronically infected with low levels of *P. relictum*, possibly for the remainder of their life (Atkinson et al. 2001). Chronic infection helps ‘amakihi develop antibodies to malarial antigens so that they do not suffer the physiological costs associated with acute infection if reinfected (Atkinson et al. 2001). ‘Amakihi response to acute infection with avian malaria has been well documented, but the impacts of chronic infection have not been studied as closely. Despite the benefit of immunity, continuing to harbor low levels of *Plasmodium* parasites may be costly. One study on ‘amakihi at a low elevation site found that chronic malarial infection did not affect reproductive success (Kilpatrick et al. 2006), but other potential impacts of chronic infection have yet to be explored.

Studies on avian malaria in other species have documented fitness reductions as a result of chronic infection. Such costs include diminished energy stores (i.e. triglycerides) (Messina et al. 2022), reduced hematocrit levels, potentially causing anemia (Schoenle et al. 2017), reduced hemoglobin levels (Schoenle et al. 2017), decreased breeding success (Knowles et al. 2010), and increased rate of telomere loss, increasing risk of age-related diseases (Asghar et al. 2015). Additionally, immunological activity associated with chronic infection can cause oxidative stress: tissue damage from reactive oxygen metabolites released in an immune response (Costantini & Dell’Omo 2006). Infection can also reduce a bird’s activity, as they become weaker and more lethargic (Adelman & Martin 2009). When less active, birds reduce fitness-enhancing behaviors such as predator evasion, foraging, and parental care; such responses have been observed in chronically infected female blue tits and multiple migratory species (Knowles et al. 2010; Risely et al. 2018). Responses to chronic malarial infection vary among bird species, and with the species of haemosporidian parasite causing avian malaria (Knowles et al. 2010;

Lachish et al. 2011; Samuel et al. 2015), so the response of one species or even population cannot be extrapolated to represent other species. Previous research has revealed various costs associated with chronic avian malaria in other species, yet the costs of chronic infection for ‘amakihi remain widely unknown.

The concentration of *P. relictum* in a chronically infected bird’s blood may also influence the bird’s physiological state. A study of Himalayan bird species demonstrated that greater levels of malarial parasites were associated with lower hemoglobin and hematocrit (Ishtiaq & Barve 2018). However, few studies have investigated the relationship between *Plasmodium* infection intensity and physiology, and none have been conducted on Hawaiian birds.

The proposed study will examine the physiological costs of chronic malarial infection in a mid-elevation population of ‘amakihi. The objectives of this study are as follows: 1) Assess the physiological status of ‘amakihi with chronic avian malaria infection and 2) Determine if the amount of *P. relictum* in ‘amakihi blood influences their physiology. With these objectives, I had the following hypotheses: H1) Chronic malarial infection in ‘amakihi will be associated with physiological costs, indicated by decreased body condition, reduced hematocrit, increased oxidative damage, and lower triglyceride levels and H2) as the quantity of *P. relictum* increases in the blood, the physiological condition of ‘amakihi will worsen. The costs of acute infection with avian malaria are well understood, whereas far less is known about the long-term effects of harboring chronic infection after surviving the acute stage. This study seeks to determine the long-term costs of avian malaria to elucidate the overall effect of this disease on wild populations.

METHODS

Study Site

This study was carried out at ʻĀinahou Ranch (19.343° N, 155.229° W), a mid-elevation site (1050 m) in Hawaiʻi Volcanoes National Park. Avian malaria is detected in about 32% of ʻamakihi at this site.

Once known as Kuaehu (Maly & Maly 2005), the area now called ʻĀinahou Ranch has a deep and rich history that long precedes the establishment of Hawaiʻi Volcanoes National Park. It is located in the ahupuaʻa of Keauhou in the Moku of Kaʻū. Historically, Keauhou was an ʻili kūpono (independent subdivision) of the ahupuaʻa of Kapāpala, but in the Māhele ʻĀina of 1848, it was designated as its own ahupuaʻa (Maly 2004; Brandt & Lopes 2023). The Keauhou trail, an ancient trade route for Kānaka Maoli to distribute resources mauka to makai, runs through ʻĀinahou. The people of Kaʻū have long been known for their toughness, as their landscape is rugged and often unforgiving (Brandt & Lopes 2023). In the ʻŌlelo Noʻeau, it is known as the land of rebels; the people were known to rebel against oppression (Pukui 1983, 183). It has also been referred to as Kaʻū Mākaha, which translates to Kaʻū of the fierce fighters (Pukui 1983, 176). Since their arrival to Hawaiʻi, Kānaka Maoli have cultivated and lived in reciprocity with these lands. The manu (birds) were so abundant that kia manu (bird catching) was common practice. Feathers were collected to create adornments for royalty and aliʻi (chiefs) such as ahuʻula (feathered capes), mahiʻole (feathered helmets), lei, and kāhili (staffs adorned with feathers) (Brandt & Lopes 2023).

Unfortunately, western contact with Hawaiʻi brought devastation to the kānaka and manu of Keauhou. Like the manu, human populations of Kaʻū dwindled following European contact, largely as a result of introduced diseases; the original population of over 10,000 kānaka plummeted to just 3,010 by 1847 (Brandt & Lopes 2023). Additionally, hapu'ū pulu (*Cibotium glaucum,* tree ferns with golden hairs at the base of their fronds) was overharvested in the 1850s as the pulu (golden hairs) were used to stuff matresses, leading to extensive damage to the habitat (*'Āinahou Ranch House and Gardens* 2004). Introduction of free-roaming cattle and livestock also led to widespread habitat loss. Kuaehu was once full of intact native mesic forest and scrubland with many plant species that are now rare or extinct, but this habitat was destroyed by grazing ruminants (*'Āinahou Ranch House and Gardens* 2004). The Keauhou trail was also colonized by the late 1800s, disrupting the distribution of resources through the ahupua'a (*'Āinahou Ranch House and Gardens* 2004). Despite these threats, kānaka and manu alike have persisted, yet another testament to the endurance and hardiness of those inhabiting Ka'ū. It is now the kuleana (responsibility) of anyone who inhabits this space to mālama 'āina (care for the land) to help restore it to its former abundance.

During the Māhele 'Āina in 1848, Keauhou was split from Kapāpala and the makai region was given to Victoria Kamamalu, granddaughter of Kamehameha I; it was then passed down to members of the Kamehameha lineage until the death of Princess Bernice Pauahi in 1884 (*'Āinahou Ranch House and Gardens* 2004). Her husband Charles Bishop sold the land to the U.S. Congress, and the land was leased to various owners for several years (*'Āinahou Ranch House and Gardens* 2004). Among these leasers was William Shipman, who may have been the one to rename Kuaehu to 'Āinahou around 1897 (Maly & Maly 2005). Most of this land was included in the establishment of the National Park in 1916, but 'Āinahou was excluded. It was leased to Herbert C. Shipman, son of William Shipman, from 1937 to 1971 (*'Āinahou Ranch House and Gardens* 2004). Shipman was a conservationist and horticulturalist and worked to integrate ‘ōhi‘a and other native trees into the gardens around the ranch house. The National Park Service acquired the land in 1971 and designated it a historical site.

Presently, ‘Āinahou Ranch consists of mostly native open mesic forest, with a mean annual temperature of 17.5 °C and mean annual precipitation of 2045 mm. The forest is dominated by ‘ōhi‘a (*Metrosideros polymorpha*) in the canopy, ‘ōhi‘a, māmane (*Sophora chrysophylla*), a‘ali‘i (*Dodonaea viscosa*), and faya (*Morella faya*) in the understory, and pukiawe (*Leptecophylla tameiameiae*), ‘ūlei (*Osteomeles anthyllidifolia*), and non-native grasses in the shrub layer. Exact population estimates have not been taken at this site, but based on field observations, ‘amakihi and warbling white-eyes (*Zosterops japonicus*) are most abundant, while ‘apapane (*Himatione sanguinea*), common myna (*Acridotheres tristis*), northern cardinals (*Cardinalis cardinalis*), yellow-billed cardinals (*Paroaria capitata*), common waxbills (*Estrilda astrild*), yellow-fronted canaries (*Crithagra mozambicus*), house finches (*Haemorhous mexicanus*), melodious laughingthrushes (*Garrulax canorus*), scaly-breasted munia (*Lonchura punctulata*), saffron finches (*Sicalis flaveola*), red-billed leiothrix (*Leiothrix lutea*) and japanese bush warblers (*Horornis diphone*) also persist in lower numbers. Potential breeding season for ‘amakihi in this area is October to June, but can vary year to year (Berger 1969).

Sample collection

Banding and sampling were conducted biweekly from May 2022 to August 2023. Birds were caught throughout the morning into early afternoon using mist nets and extracted for processing every 30 minutes. During processing, first-capture ‘amakihi were banded with a USGS aluminum numerical band, measured for tarsus, bill, and wing length, weighed, and checked for molting, fault bars, and lesions. Birds were checked for breeding characteristics, namely a brood patch in females, or a cloacal pertuberence in males. They were then aged and sexed based on plumage, breeding characteristics, and size (Paxton et al. 2016b). Age was divided into three classes: juvenile plumage, indicative of hatch year birds, formative plumage, characteristic of hatch year and some second-year birds, and definitive plumage, characteristic of some after hatch year and after second year birds. In addition, a blood sample (up to 75 µL, <1% body mass) was taken by inserting a precision glide needle gently into the brachial vein and allowing the blood to wick into a capillary tube. Samples were stored on ice until the end of the day. Then samples were spun down in a centrifuge to separate plasma from the erythrocytes, and total blood and total red blood cells were measured in the capillary tube using calipers. Hematocrit was calculated as the proportion of total blood that is red blood cells. Red blood cells were extracted into a microcentrifuge tube with 600 µL tris-ethylenediaminetetraacetic acid (TEN; 10 mM Tris, 10 mM NaCl, 2 mM EDTA, pH 8.0, Atkinson 2020) buffer solution. In the case that the total blood was less than 10 mm, plasma and red blood cells were not separated, rather stored together with 300 µL TEN buffer solution. Otherwise, plasma was extracted into a microcentrifuge tube, and stored at -80°C.

Recaptured ‘amakihi had their band number recorded and were processed in the same way as first-captured birds. Only data from the capture date on which the most blood was collected was used for analysis, so recaptured birds are only factored once into the dataset.

Classifying life stage

Age and breeding efforts can play a significant role in avian physiology (Vézina & Williams 2003; Alonso-Álvarez et al. 2010; Bize et al. 2014; Delhaye et al. 2016; Brown et al. 2021). Breeding status (breeding or non-breeding) was determined based on breeding characteristics; ‘amakihi were considered actively breeding if they had a brood patch or a medium or large cloacal pertuberence. Age was simplified for molting birds to be either formative plumage or definitive plumage, and juveniles were not included in the study because no juvenile ‘amakihi tested positive for avian malaria.

Genetic Sexing

Subjective field assessments of sex can often be incorrect, so the sex of each bird was confirmed using genetic sexing methods. 1 μL of extracted DNA was mixed with 14 μL master mix consisting of Ddh20, Taq, and forward and reverse primers. DNA was amplified using a C1000 Touch Thermocycler with a CFX96 Real-time System (Bio-Rad Laboratories) programed to the following cycles: 94°C for 4 minutes, 37 cycles of heating and cooling from 94°C for 30 seconds to 57°C to 50°C for 45 seconds, 72 °C for 45 seconds, and lastly 74°C for 5 minutes. 5 μL of amplified DNA with 1 μL dye for each sample along with 2 μL of DNA ladder was then run through gel electrophoresis using 1.5% gel at 100 volts for 5 minutes. For males, one band appears around 500 base pairs (bp), whereas for females, two bands appear: one at 500 bp and another at 300 bp (Purwaningrum et al. 2019).

Determining disease status

The disease status of each ‘amakihi was determined by quantifying parasitemia using a quantitative polymerase chain reaction (qPCR) assay. GRW4-specific primers were used to target the cytochrome b gene found in *P. relictum* (Zehtindjiev et al. 2008) along with a highly specific fluorescent probe designed by Paxton et al. (2023). DNA was extracted from blood samples using the DNeasy Blood and Tissue Kit (Qiagen cat # 69504). Proteinase K first lyses the samples, and then the samples are centrifuged to allow DNA to selectively bind to the

DNeasy membrane as contaminants pass through. Lastly, two wash steps remove any remaining contaminants or enzyme inhibitors and the purified DNA is stored in buffer solution.

Prior to qPCR, the genomic DNA of each extracted sample was quantified using a Broad Range Qubit fluorometer (Invitrogen). Samples with a DNA concentration less than 22 ng/µL were re-extracted. Using the concentration of genomic DNA, samples were diluted to achieve a standard concentration of 20 ng/µL. In a Bio-Rad 96 well, thin-walled plate (Cat #HSP9655), samples were run in triplicate by mixing 5 µL dilute genomic DNA with 10 µL iTac Universal Probe Supermix, 1.5 µL of each primer, 0.5 µL of GRW4 probe, and 1.5 µL PCR-grade water. For each sample, the presence of bird DNA was also tested for in singlet by mixing 5 µL dilute genomic DNA with 10 µL iTac Universal Probe Supermix, 0.6 µL of each primer, 0.5 µL of 18sAVHyb probe, and 3.3 µL PCR-grade water. In addition to the genomic DNA samples, each plate also included a positive and negative control, and PCR-grade water as a non-template control (NTC). A C1000 Touch Thermocycler with a CFX96 Real-time System (Bio-Rad Laboratories) was used to carry out qPCR using the following cycles: 95°C for 5 minutes followed by 50 cycles of 95°C for 5 seconds and 59°C for 30 seconds. The C_T (Cycle Threshold) value of each sample was determined, and the overall C_T value for each sample was determined to be the median of the three triplicate samples; if one of the three did not amplify or was an outlier (not within 1 C_T value), it was excluded from C_T value concentration. If only one of three triplicates amplified, the sample was determined inconclusive and re-run. Samples with a C_T value of 0 were considered undetermined, meaning the bird is either uninfected or has very low parasitemia levels that are not detectable by qPCR. Those with a C_T value between 20 and 50 were considered positive. A lower C_T value (excluding values of 0) is indicative of a higher concentration of *P. relictum* in the bird‘s blood.

Recaptured ‘amakihi could be confirmed as chronically infected during a given capture event if they had been positive in the capture event before and after. The cutoff between acute and chronic infection in terms of C_T value has yet to be determined. However, the likelihood of catching an ‘amakihi at the very beginning stages of acute infection when parasitemia is relatively low is slim due to the short time frame of this stage, and acutely infected birds have significantly reduced activity levels that make it highly unlikely to capture them (Lapointe et al. 2012; Schoener et al. 2014). However, capturing a bird multiple times is the only way to confirm that they are chronically infected without having measured malarial antibodies.

Oxidative Stress

Levels of oxidative stress in ‘amakihi were determined by measuring reactive oxygen metabolites (ROMs) using the d-ROMs test kit (Diacron International, MC003) following the methods described in Schoenle et al. (2017) with some modifications. ROMs are hydroperoxides generated when reactive oxygen species attack organic substrates. Thus, the amount of ROMs in the plasma is proportionate to the amount of tissue damage a bird has experienced. A 1:100 solution of chromogen to sodium acetate buffer created to establish the working solution. 4 µL ‘amakihi plasma was then added in duplicate to a clear, flat-bottomed 96-well plate. Additionally, 4 µL of internal control (canary plasma) was added in duplicate as well as a blank (dionized water) and calibrator (Diacron International, MC030) added in quadruplicate to. Then 200 µL of working solution was added to each well and slowly pipette mixed. The plate was incubated at 37°C for 75 minutes. The plate was then scanned for absorbance on a SpectraMax M2 Microplate Reader (Molecular Devices) at 492 nm. The average of duplicate and quadruplicate samples was used for calculations. Wells that did not react, indicated by absorbance values that did not exceed two standard deviations of the mean blank absorbance, were not included. ROMs were calculated using the kit's equation: ((Absorbance of Sample-Absorabance of Blank)/(Absorbance of Calibrator-Absorbance of Blank))*(Concentration of Calibrator) where the Calibrator Concentration=290 UCARR (1 UCARR=0.08 mg H_2O_2/dL).

Triglycerides

Since d-ROMs measures damage to lipids by reactive oxygen species, it is critical that lipids also be measured to determine the proportion of damaged lipids to total lipids (Pérez-Rodríguez et al. 2015b). Triglycerides are also a good indicator of energetic balance in individuals. Therefore, triglycerides were quantified using the LabAssay Triglyceride kit (FUJIFILM) based on the methods described in Messina et al. (2020). Two dilutions of the standard solution (300 mg/dL triglycerides) were created to get concentrations of 100 mg/dL and 200 mg/dL. The color reagent was prepared by dissolving one vial of chromogen substrate in 105 mL of buffer solution. 2 µL of ‘amakihi plasma, internal control (yellow fronted canary plasma), or triglyceride standard (5 different standard concentrations) were added in duplicate to a clear, flat-bottomed 96-well plate. Then 300 µL of color reagent was added to each well and slowly pipette mixed. The plate incubated for 15 minutes and was then scanned for absorbance on a SpectraMax M2 Microplate Reader (Molecular Devices) at 595 nm. A standard curve was developed using the standards and their respective absorbance values, the slope of this curve was used to calculate triglycerides for each well. The average of duplicates was taken to determine each sample's triglyceride content.

Statistical analysis

Statistical tests were conducted using RStudio version 2021.09.0. To assess the influence of infection on ‘amakihi physiology, a linear mixed model was run for each physiological parameter- oxidative damage, triglycerides, hematocrit, and body condition- using the *lme4* package. For all tests including C_T value, C_T was square root transformed to achieve a normal distribution.

Sex, breeding status, and age have all shown to impact hematocrit in other bird species, and often interact in their effect (Villegas et al. 2004; Owen et al. 2005; Wagner et al. 2008; Brown et al. 2021). The global linear mixed model included hematocrit as the response variable, disease status as a fixed effect and breeding status, sex, and age as random effects. An analysis of variance (ANOVA) was used to compare this global model with parsed-down models and a null model including only random effects (Table 1). The top model was that with disease status as a fixed effect and age as a random effect as it had the greatest log likelihood and lowest AIC. Thus, this model was used for the analysis. Lastly, a correlation analysis was conducted to examine the relationship between hematocrit and C_T value.

Table 1. Ranking of top hematocrit linear mixed models as well as global and null models with disease status as a fixed effect, and age, sex, and breeding status as random effects. Rankings are based on AIC.

Model	npar	AIC	BIC	logLik	Rank
Disease Status + (1|Sex) + (1|Age) + (1|Breeeding) (GLM)	6	-866.79	-845.86	439.40	4
1 + (1|Sex) + (1|Age) + (1|Breeeding) (Null)	5	-867.95	-850.50	438.97	3
Disease Status + (1|Age)	4	-870.79	-856.84	439.40	1
Disease Status + (1|Age) + (1|Sex)	5	-868.79	-851.35	439.40	2

ROMs were square root transformed to achieve a normal distribution. Triglycerides were included as a covariate in the model; because dROMs is measuring damage to lipids, it is crucial to take total lipids into account (Pérez-Rodríguez et al. 2015a). Breeding activity and age can also influence oxidative stess (Alonso-Álvarez et al. 2010; Bize et al. 2014; Delhaye et al. 2016).

The global linear mixed model included square root transformed ROMs as a response variable, disease status as a fixed effect and triglycerides, breeding status, sex, and age as random effects. An ANOVA was used to compare the global model with parsed-down models and a null model with only random effects (Table 3). The top model used for analysis was that with disease status as a fixed effect and triglycerides as a random effects as it had one of the greatest log likelihoods and had the lowest AIC by far. Lastly, a correlation analysis was conducted to examine the relationship between ROMs and C_T value.

Table 2. Ranking of top dROMs linear mixed models as well as null model with disease status as a fixed effect, and age, sex, breeding status, and triglycerides as random effects. Rankings are based on AIC.

Model	npar	AIC	BIC	logLik	Rank
Disease Status + (1|Sex) + (1|Age) + (1|Breeding Status) + (1|Triglycerides)	7	848.45	871.18	-417.22	2
1 + (1|Sex) + (1|Age) + (1|Breeding Status) + (1|Triglycerides)	6	848.50	867.98	-418.25	3
Disease Status + (1|Triglycerides)	4	842.45	855.43	-417.22	1

Triglycerides were logarithmically transformed to achieve a normal distribution. Along with sex, breeding status, age, and time of day can also influence triglyceride levels (Vézina & Williams 2003; Owen et al. 2005; Rodríguez et al. 2006; KEÇECİ & ÇÖL 2011; Sepp et al. 2012). The global linear mixed model included log transformed triglycerides as a response variable, disease status as a fixed effect and breeding status, sex, age, and time of bleeding as random effects. An ANOVA was used to compare this global model with parsed-down models and a null model with only random effects (Table 3). The top model was that with disease status as a fixed effect and age and time as random effects as it was among the three with the greatest log likelihood and had the lowest AIC. Thus, this model was used for the analysis. Lastly, a correlation analysis was conducted to examine the relationship between triglycerides and C_T value.

Table 3. Ranking of top triglycerides linear mixed models as well as null model. with disease status as a fixed effect, and age, sex, breeding status, and time of day as random effects. Rankings are based on AIC.

Model	npar	AIC	BIC	logLik	Rank
Disease Status + (1 | Sex) + (1 | Age) + (1 | Breeding Status) + (1 | Time) **(GLM)**	7	73.332	97.151	-29.666	5
1 + (1 | Sex) + (1 | Age) + (1 | Breeding Status) + (1 | Time) **(Null)**	6	72.705	93.121	-30.352	4
Disease Status + (1 | Age) + (1 | Time)	5	69.824	86.837	-29.912	1
Disease Status + (1 | Age) + (1 | Breeding Status) + (1 | Time)	6	71.332	91.748	-29.666	2
Disease Status + (1 | Time)	4	71.471	85.081	-33.727	3
Disease Status	3	73.454	83.662	-33.727	6

Body condition was determined by dividing the mass of each bird by their wing length. Sex is likely an important factor in body condition since ‘amakihi are sexually dimorphic, with males typically larger than females (Paxton et al. 2016b). Breeding is also associated with an increased metabolic rate in avian species which may result in mass reductions (Bryant 1988; Nilsson & Råberg 2001; Nord & Williams 2015). The global linear mixed model included body condition as a response variable, disease status as a fixed effect and breeding status, sex, and age as random effects. An ANOVA was used to compare the global model with parsed-down models and a null model with only random effects (Table 4). The top model used for analysis was that with disease status as a fixed effect and sex as a random effect as it was among the highest of log likelihoods and had the lowest AIC. Lastly, a correlation analysis was conducted to examine the relationship between body condition and C_T value.

Table 4. Ranking of top body condition models as well as global model and null model with disease status as a fixed effect, and sex, age, and breeding status as random effects. Rankings are based on AIC.

Model	npar	AIC	BIC	logLik	Rank
Disease Status + (1|Sex) + (1|Age) + (1|Breeding) (GLM)	6	-1362.4	-1341.7	687.21	4
1 + (1|Sex) + (1|Age) + (1|Breeding) (Null)	5	-1363.1	-1345.9	686.55	3
Disease Status + (1|Sex) + (1|Breeding)	5	-1364.4	-1347.2	687.21	2
Disease Status + (1|Sex)	4	-1366.4	-1352.6	687.21	1

After running these models comparing all positive individuals with all undetermined individuals, the top model for each parameter was re-run using only positive birds that were confirmed to be chronically infected with all undetermined birds. If these results were the same, then it was assumed that the results of the models including all positive birds could be extrapolated to apply to chronically infected ‘amakihi as well.

Permitting

The research for this study has been approved by UHH IACUC (protocol #18-2916-4) and is permitted under USGS bird banding permit #23064 , Hawai‘i State Wildlife Research Permit # WL21-08, and Hawai‘i Volcanoes National Park Permit # HAVO-2022-SCI-0015.

RESULTS

Infection rate

Blood samples were collected from a total of 242 ‘amakihi (63 positive, 179 undetermined). Of the 63 positive ‘amakihi, 32 were confirmed as chronically infected. Although most of the remaining 31 positive ‘amakihi were most likely chronically infected, there were not enough recapture events to confirm this. The mean amplification efficiency of all qPCR runs was 1.903 +/- 0.001 (SE), and inter-plate variation was 2.2%.

Hematocrit

Hematocrit was measured in 242 ‘amakihi from which blood samples were collected (63 infected, 179 undetermined). There was no relationship between disease status and hematocrit levels (p=0.400, Figure 1). Hematocrit averaged 0.489 for infected ‘amakihi, 95 % CI [0.471, 0.506], and 0.484, 95 % CI [0.438, 0.529] for undetermined ‘amakihi. A negligible amount of variance in hematocrit could be explained by disease status (<0.0005 %). About 0.0008 % of variance was explained by age (random effects). The overall model only explained 0.16 % of variation in hematocrit. Among 63 ‘amakihi that tested positive for avian malaria, there was no significant correlation between C_T and hematocrit (p=0.2768, Figure 2). Results did not change when models were run with only ‘amakihi that were confirmed to be chronically infected.

Oxidative Stress

ROMs were measured in 210 ‘amakihi (52 positive, 158 undetermined). Inter-plate variation for the assay was 11.1 %. There was a significant positive correlation between triglycerides and ROMs (r(188)=0.2506, p=0.0005, Figure 3). Although ROMs were slightly higher in positive ‘amakihi than undetermined, this difference was not signficant (p=0.155, Figure 4). For positive ‘amakihi, average ROMs was 101.00 UCARR, 95% CI [89.11, 114.49], while for undetermined ‘amakihi, it was 91.01 UCARR, 95% CI [84.09, 98.01]. Most variation in ROMs was explained by random effects, namely variation in triglycerides (368.5 %). Just 5.2 % of variation in ROMs was explained by disease status. The overall model explained 112 % of variation in ROMs. There was no correlation between ROMs and C_T value (p=0.475, Figure 5).

Results did not change when models were run with only ‘amakihi that were confirmed to be chronically infected.

Triglycerides

Triglyceride levels were measured in 222 ‘amakihi (61 positive, 161 undetermined) Inter-plate variation for the assay was 14.9 %. There was a positive correlation between time of day and triglycerides ($r(220)=0.21$, $p=0.002$, Figure 6). There was no difference in triglyceride levels between positive and undetermined ‘amakihi ($p=0.2825$, Figure 7). Triglycerides averaged 235.10 mg/dL for positive ‘amakihi, 95 % CI [160.77, 340.36], and 223.63 mg/dL for undetermined ‘amakihi, 95 % CI [117.92, 424.11]. 0.04 % of variance in triglycerides was explained by disease status. Some variance (2.66 %) came from random effects (age and time). The overall model explained 5.8 % of variation in triglycerides. There was no relationship between C_T value and triglycerides ($p=0.3577$, Figure 8). Results did not change when models were run with only ‘amakihi that were confirmed to be chronically infected.

Body Condition

Body condition was recorded for a total of 232 ‘amakihi (62 positive, 170 undetermined). There was no difference in body condition between positive and undetermined ‘amakihi ($p=0.248$, Figure 9). Body condition was 0.208 g/mm wing length for infected ‘amakihi, 95 % CI [0.199, 0.216] and 0.205 g/mm wing length for undetermined ‘amakihi, 95 % CI [0.124, 0.287]. A negligible amount of variance in body condition could be explained by disease status (<0.0005 %). Sex (random effect) also explained very little variance in body condition (<0.0005 %). The overall model only explained 0.016 % of variation in hematocrit. There was no correlation between C_T value and body condition ($p=0.3195$, Figure 10).

Body condition results did differ when the model was run only with positive birds that were confirmed to be chronically infected. Body condition was greater in chronically infected ‘amakihi than in undetermined ‘amakihi ($p=0.0287$, Figure 11). However, there was no correlation between C_T value and body condition for confirmed chronically infected ‘amakihi ($p=0.4614$).

DISCUSSION

Overall, these results demonstrate that hematocrit, oxidative stress, and triglycerides do not differ between undetermined and positive ‘amakihi at ‘Āinahou Ranch. Body condition did differ when eliminating any birds that were not confirmed to be chronically infected from the analysis, and was slightly higher in chronically infected ‘amakihi. These results suggest that chronic infection does not have a physiological toll on Hawai‘i ‘amakihi. Infected birds had a broad range of C_T values, indicating differing levels of parasitemia. However, parasitemia as indicated by C_T value did not affect hematocrit, triglycerides, oxidative stress, nor body condition.

There is not a consensus on the C_T cutoff between chronic and acute infection. Some ‘amakihi in this study did have C_T values in the 20’s, which others may have considered acute (Videvall et al. 2021), but it has not yet been determined what C_T values correspond with what stage of infection. Although captured birds are highly unlikely to be in the acute stage of infection upon capture, this could only be confirmed for recaptured birds. Although it can likely be assumed most if not all positive birds are chronically infected, analyses were rerun with only definitively chronically infected birds to confirm if the results can be extrapolated to apply for chronic infection. In most cases they could be except in the case of body condition.

Hematocrit

Reduced hematocrit and anemia are consistently associated with acute avian malaria across species (LaPointe et al. 2012; Schoener et al. 2014; Names et al. 2022). While anemia is suspected to be the primary cause of mortality at the acute stage on infection for ‘amakihi (Atkinson et al. 2000), this study suggests that reduced hematocrit levels do not persist into the chronic infection stage. Only one ‘amakihi, a definitive plumage uninfected male, had hematocrit levels low enough to be considered anemic (19%). Based on the lack of difference in hematocrit between infected and undetermined ‘amakihi, neither group seems more prone to anemia. Other birds chronically infected with avian malaria have shown reduced hematocrit levels. Chronically infected redwinged blackbirds, for example, have lower hemoglobin and hematocrit, but also increased red blood cell production, likely to offset the destruction of red blood cells by *Plasmodium* species (Schoenle et al. 2017). Chronically infected ‘amakihi that host low levels of *P. relictum* either do not suffer greater damage to red blood cells, or if they do, increase red blood cell production to offset damage to red blood cells caused by the haemosporidian parasite.

Oxidative Stress

The lack of difference in ROMs between positive and undetermined ‘amakihi is further evidence of tolerance to chronic infection. It seems that either ‘amakihi are not mounting an immune response against this low-level parasitemia, but rather offsetting costs associated with the parasites, or they are counteracting oxidative damage by increasing antioxidant circulation, likely by foraging more (Delhaye et al. 2016). This could be tested by measuring antibody levels or pro-oxidants in the blood. Studies of other species have similarly found that chronic infection is not a cause for oxidative stress (Schoenle et al. 2017). Oxidative stress can have serious negative impacts on fitness, reducing lifespan, growth, and reproduction (reviewed in Costantini 2016). The lack of oxidative stress for positive ‘amakihi suggests that the fitness of chronically infected ‘amakihi is not diminished by such factors.

Triglycerides

Avian triglyceride levels tend to start lowest in the morning following overnight fasting, and then increase throughout the day as they eat (Rodríguez et al. 2006; Sepp et al. 2012). The positive corellation between time and triglycerides in ‘amakihi suggests that they too follow this trend. The lack of difference in triglycerides between infected and undetermined ‘amakihi suggests that chronic infection either is not energetically costly, or that ‘amakihi are able to offset any energetic costs, perhaps by reducing movement or by increasing foraging.

Body Condition

Mass is often an effective predictor of survival in avian species (Covas et al. 2002). These results demonstrating that chronic infection does not reduce body condition, along with the lack of other detectable physiological costs, contributes to a growing body of evidence that chronic malarial infection likely does not have significant costs to ‘amakihi fitness.

It is unexpected that body condition is greater in chronically infected birds. One possible explanation is that only birds of greater body condition are likely to survive acute infection, and so birds that survive this challenge are biased towards being of better condition. The poor-condition birds would be more likely to subcome to mortality. Another factor to consider is that the models for body condition of chronically infected birds had singularities as a result of the drastically reduced and imbalanced sample size. The model with disease as a fixed effect and sex as a random effect only explained 0.013 % of variation in body condition. This suggests that other factors not accounted for in this study are driving variation in body condition moreso than disease status. The relationship between chronic infection and body size requires further investigation with a greater sample size before definitive conclusions can be drawn.

C_T *value and physiology*

None of the phsyiological variables assessed- hematocrit, ROMs, triglycerides, nor body condition- were significantly correlated with C_T value. Thus, the intensity of chronic infection does not seem to impact physiological stress levels in ‘amakihi. This demonstrates a level of tolerance, whereby the ‘amakihi offset costs of chronic infection. Further research into the immune activity of chronically infected birds testing for the presence of malaria-specific antibodies would further elucidate their response to chronic infection. Based on these results, the quantity of *P.* relictum does not appear to affect the physiology of chronically infected ‘amakihi.

Limitations

The prevalence rate of avian malaria in ‘amakihi was 26 % in this analysis. It is important to note that the overall infection rate may be higher, as this is only accounting for birds that become infected and survive. As the ‘Āinahou project continues, a long-term dataset will be established that will allow for survival estimates. Thus, an important next step is to determine the survival rate and overall *P. relictum* infection rate for ‘amakihi at this site. This research lays the groundwork for continual physiological monitoring of this ‘amakihi population. One such topic that could be addressed using survival estimates is if there are physiological predictors for whether or not a bird survives acute infection.

There are far more males (n=175) included in this study than females (n=35), as male ‘amakihi are generally more active and therefore more likely to fly into the nets (Smetzer et al. 2022). Additionally, the models that only included chronically infected birds often had issues with singularities. It would be beneficial to re-run these analayses with a larger and more balanced sample size to see if the results remain the same with more powerful models.

Lastly, this study assumes that ‘amakihi infected with avian malaria remain chronically infected for the remainder of their lives. Thus, birds that did not have detectable *P. relictum* in their blood were assumed to have never been infected with the parasite. A more complete dataset at ‘Āinahou will reveal if this assumption is true by monitoring infected birds over several years.

CONCLUSIONS

Most studies on the direct effects of avian malaria in ‘amakihi have focused on acute infection using captive challenge studies. This study is one of the first to assess the long-term impacts of avian malaria in a wild population. The only other study of chronic infection in Hawai‘i ‘amakihi found that reproductive success was not impacted by chronic avian malaria (Kilpatrick et al. 2006). In fact, in Kilpatrick‘s study, which was also carried out at ‘Āinahou Ranch, pairs with chronically infected males had even higher nesting success and nestlings that were more likely to be resighted a year later. This could be interpreted one of two ways: 1) healthier ‘amakihi are more likely to survive acute infection; thus, chronically infected birds would have greater fitness overall because the less fit birds would have died from infection or 2) ‘amakihi with greater fitness are more prone to infection because they move around more or are more attractive to mosquitos. Though most physiological parameters did not differ between undetermined and chronically infected birds, mass was higher in chronically infected birds.

Further research on the survival, acitvity, and reproduction of chronically infected ‘amakihi in this area could continue to investigate the possibility of greater fitness in chronically infected birds.

This study contributes to a growing body of evidence that ‘amakihi reslience against avian malaria is not just in the acute stage, but in the chronic stage as well. Based on these results, combined with previous work by Kilpatrick et al. (2006), there may not be long-term costs to fitness associated with chronic avian malaria in this population of ‘amakihi. Thus, studying survivors of infection could prove critical in elucidating factors that shape their resilience. It is hypothesized that chronically infected ‘amakihi carry genes for malarial resistance, so methods of facilitating the evolution of these genes in less resilient populations are being considered (Kilpatrick 2006; Samuel et al. 2020). It is possible that the reslience of ‘amakihi also stems from their generalist diet, and that certain microbes in their gut microbiota stimulates antibody production against *P. relictum* and increases chances of survival (Navine et al. 2022). Finding ways to help birds survive the acute stage of infection is critical in saving endemic Hawaiian forest birds from extinction. The ‘amakihi serves as a beacon of hope in avian conservation; it seems that the ‘amakihi of Keauhou, like the kānaka throughout Ka‘ū, persist and even thrive despite the challenges they face.

APPENDIX

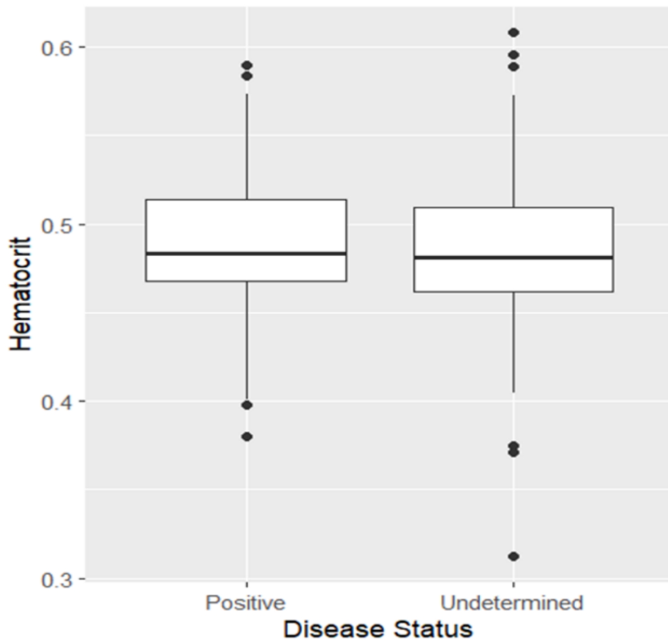

Figure 1. Hematocrit levels based on disease status in ‘amakihi. There is no difference in hematocrit between positive and undetermined ‘amakihi (p=0.400).

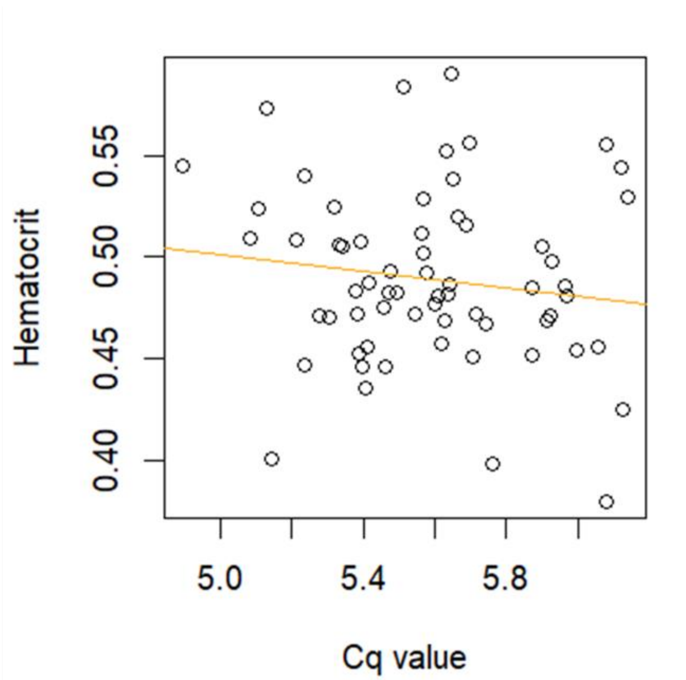

Figure 2. Hematocrit levels plotted against square root transformed Cq value. There is no relationship between hematocrit and Cq value for infected ‘amakihi (p=0.2768).

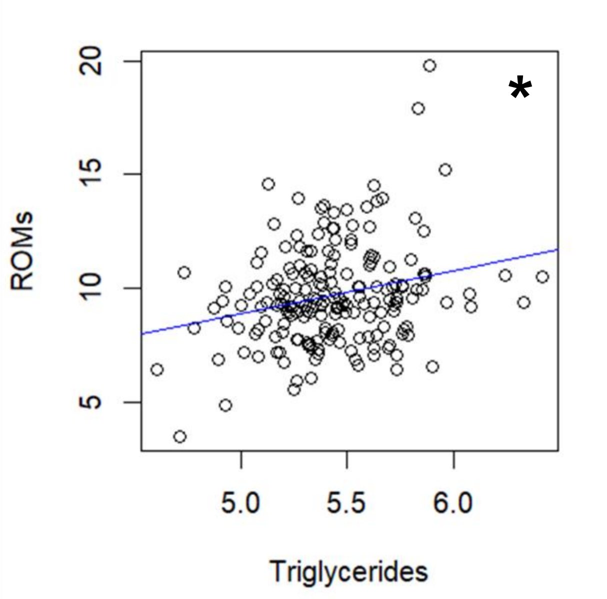

Figure 3. Square root transformed ROMs plotted against log transformed triglycerides. There is a significant positive correlation between the two (r(188)=0.2506, p=0.0005).

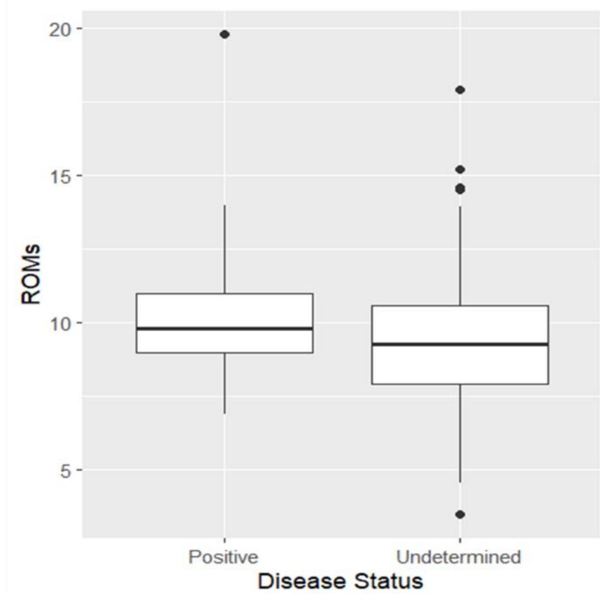

Figure 4. Square root transformed ROMs levels based on disease status in ‘amakihi. There is no significant difference in ROMs between positive and undetermined ‘amakihi (p=0.155).

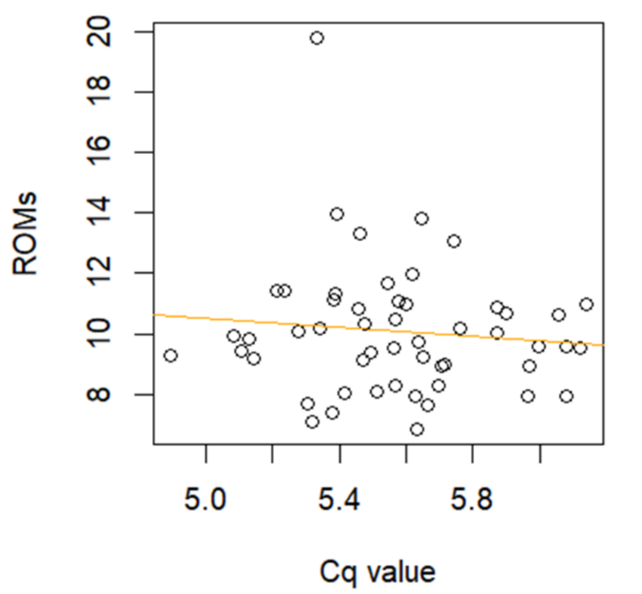

Figure 5. Square root transformed ROMs levels plotted against square root transformed Cq value. There is no relationship between ROMs and Cq value for infected ‘amakihi (p=0.475).

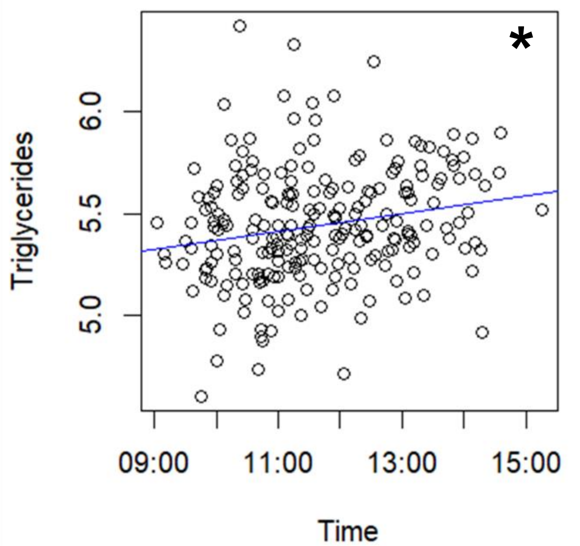

Figure 6. Log transformed triglyceride levels based on time of day blood sample was taken (r(220)=0.21, p=0.0021).

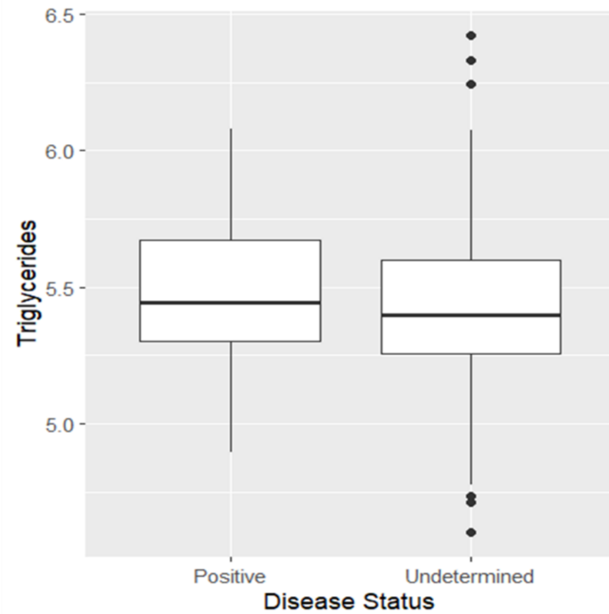

Figure 7. Log transformed triglyceride levels based on disease status in ʻamakihi. There is no difference in triglycerides between positive and undetermined ʻamakihi (p=0.2825).

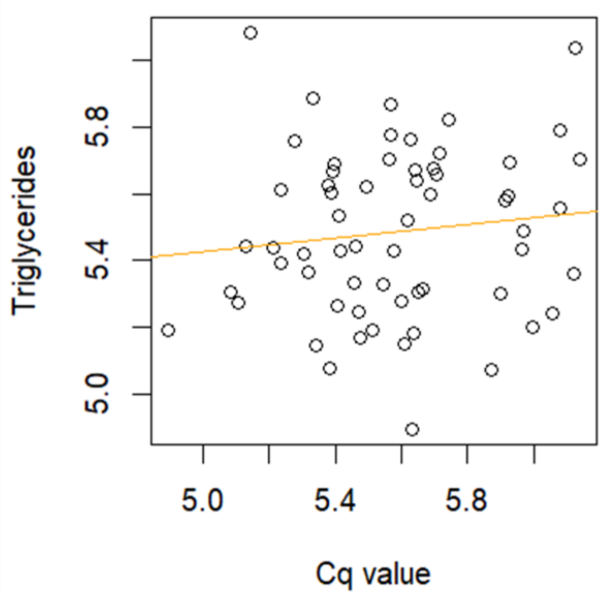

Figure 8. Log transformed triglyceride levels plotted against square root transformed Cq value. There is no relationship between triglycerides and Cq value for infected ʻamakihi (p=0.3577).

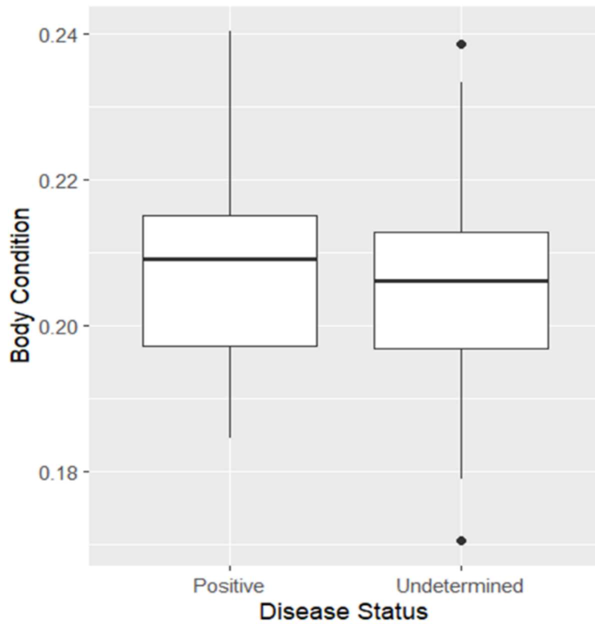

Figure 9. Body condition by disease status in all infected ‘amakihi. There is no significant difference in body condition between positive and undetermined ‘amakihi (p=0.248).

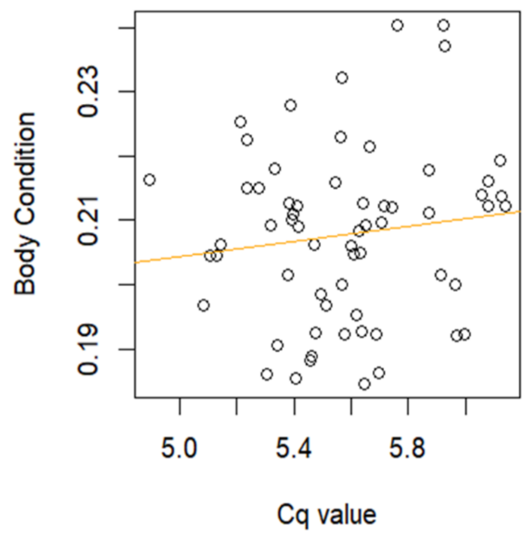

Figure 10. Body condition plotted against square root transformed Cq value. There is no relationship between body condition and Cq value for infected ‘amakihi (p=0.3195).

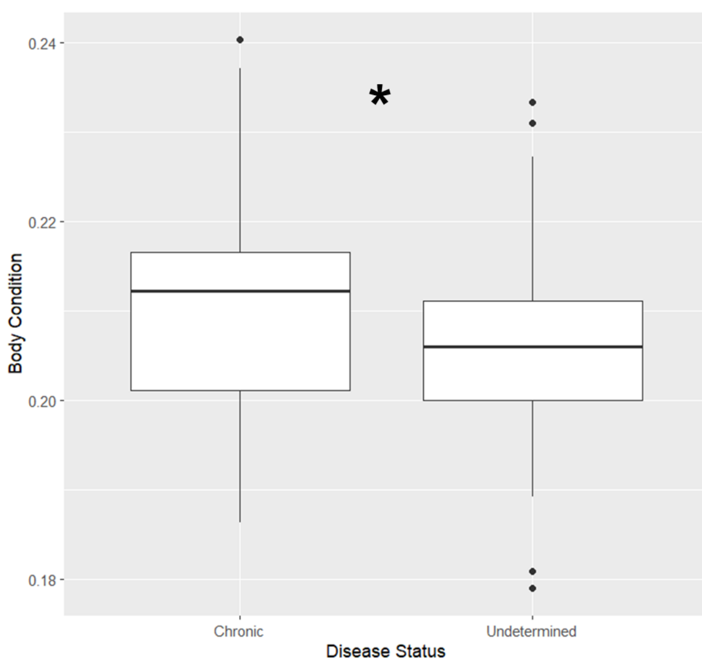

Figure 11. Body condition by disease status in ‘amakihi when only including positive ‘amakihi that were confirmed to have chonic malarial infection. Body condition is greater in chronically infected ‘amakihi (p=0.0287).

WORKS CITED

Adelman JS, Martin LB. 2009. Vertebrate sickness behaviors: Adaptive and integrated neuroendocrine immune responses. *Integrative and Comparative Biology* **49**:202–214.

Alonso-Álvarez C, Pérez-Rodríguez L, García JT, Viñuela J, Mateo R. 2010. Age and Breeding Effort as Sources of Individual Variability in Oxidative Stress Markers in a Bird Species. *Physiological and Biochemical Zoology* **83**:110–118. The University of Chicago Press.

Amante-Helweg VLU, Conant S. 2009. Hawaiian Culture and Forest Birds. Pages 59–79 in Pratt TK, Atkinson CT, Banko PC, Jacobi JD, Woodworth BL, editors. Conservation Biology of Hawaiian Forest Birds: Implications for Island Avifauna. Yale University Press.

Asghar M, Hasselquist D, Hansson B, Zehtindjiev P, Westerdahl H, Bensch S. 2015. Hidden costs of infection: Chronic malaria accelerates telomere degradation and senescence in wild birds. *Science* **347**:436–438. American Association for the Advancement of Science.

Atkinson CT. 2020. Use of whole blood samples preserved in DNA lysis buffer for serological detection of avian malaria in Hawaiian forest birds. Available from http://hdl.handle.net/10790/5287 (accessed June 2, 2024).

Atkinson CT, Dusek RJ, Lease JK. 2001. Serological responses and immunity to superinfection with avian malaria in experimentally-infected Hawaii Amakihi. *Journal of Wildlife Diseases* **37**:20–27.

Atkinson CT, Dusek RJ, Woods KL, Iko WM. 2000. Pathogenicity of avian malaria in experimentally-infected Hawaii Amakihi. *Journal of Wildlife Diseases* **36**:197–201.

Atkinson CT, Woods KL, Dusek RJ, Sileo LS, Iko WM. 1995. Wildlife disease and conservation in Hawaii: Pathogenicity of avian malaria (Plasmodium relictum) in experimentally infected Iiwi (Vestiaria coccinea). *Parasitology* **111**:S59–S69. Cambridge University Press.

Berger AJ. 1969. The breeding season of the Hawaii ’amakihi. Bernice P. Bishop Museum, Honolulu, HI.

Bize P, Cotting S, Devevey G, van Rooyen J, Lalubin F, Glaizot O, Christe P. 2014. Senescence in cell oxidative status in two bird species with contrasting life expectancy. *Oecologia* **174**:1097–1105.

Brandt L, Lopes SK. 2023. Cultural Impact Assessment for the Kapāpala Koa Canoe Management Area. 39580.00. ASM affiliates, Kapāpala Ahupuaʻa, Kaʻū District, Island of Hawaiʻi.

Brown TJ, Hammers M, Taylor M, Dugdale HL, Komdeur J, Richardson DS. 2021. Hematocrit, age, and survival in a wild vertebrate population. *Ecology and Evolution* **11**:214–226.

Bryant DM. 1988. Energy Expenditure and Body Mass Changes as Measures of Reproductive Costs in Birds. *Functional Ecology* **2**:23–34. [British Ecological Society, Wiley].

Costantini D. 2016. Oxidative stress ecology and the d-ROMs test: facts, misfacts and an appraisal of a decade’s work. *Behavioral Ecology and Sociobiology* **70**:809–820.

Costantini D, Dell’Omo G. 2006. Effects of T-cell-mediated immune response on avian oxidative stress. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* **145**:137–142.

Covas R, Brown CR, Anderson MD, Brown MB. 2002. Stabilizing selection on body mass in the sociable weaver Philetairus socius. *Proceedings of the Royal Society of London. Series B: Biological Sciences* **269**:1905–1909. Royal Society.

Delhaye J, Jenkins T, Christe P. 2016. Plasmodium infection and oxidative status in breeding great tits, Parus major. *Malaria Journal* **15**:531.

Eggert LS, Terwilliger LA, Woodworth BL, Hart PJ, Palmer D, Fleischer RC. 2008. Genetic structure along an elevational gradient in Hawaiian honeycreepers reveals contrasting evolutionary responses to avian malaria. *BMC Evolutionary Biology* **8**:315.

Foster JT, Woodworth BL, Eggert LE, Hart PJ, Palmer D, Duffy DC, Fleischer RC. 2007. Genetic structure and evolved malaria resistance in Hawaiian honeycreepers. *Molecular Ecology* **16**:4738–4746.

‘Āinahou Ranch House and Gardens. 2004. National Park Service Cultural Landscapes Inventory. Hawai’i Volcanoes National Park.

Ishtiaq F, Barve S. 2018. Do avian blood parasites influence hypoxia physiology in a high elevation environment? *BMC Ecology* **18**:15.

KEÇECİ T, ÇÖL R. 2011. Haematological and biochemical values of the blood of pheasants (Phasianus colchicus) of different ages. *Turkish Journal of Veterinary & Animal Sciences* **35**:149–156.

Kilpatrick AM. 2006. Facilitating the evolution of resistance to avian malaria in Hawaiian birds. *Biological Conservation* **128**:475–485.

Kilpatrick AM, LaPointe DA, Atkinson CT, Woodworth BL, Lease JK, Reiter ME, Gross K. 2006. Effects of chronic avian malaria (Plasmodium relictum) infection on reproductive success of Hawaii Amakihi (Hemignathus virens). *The Auk* **123**:764–774.

Knowles SCL, Palinauskas V, Sheldon BC. 2010. Chronic malaria infections increase family inequalities and reduce parental fitness: experimental evidence from a wild bird population. *Journal of Evolutionary Biology* **23**:557–569.

Lachish S, Knowles SCL, Alves R, Wood MJ, Sheldon BC. 2011. Fitness effects of endemic malaria infections in a wild bird population: the importance of ecological structure. *Journal of Animal Ecology* **80**:1196–1206.

LaPointe DA, Atkinson CT, Samuel MD. 2012. Ecology and conservation biology of avian malaria: Ecology of avian malaria. *Annals of the New York Academy of Sciences* **1249**:211–226.

Lapointe DA, Atkinson CT, Samuel MD. 2012. Ecology and conservation biology of avian malaria. *Annals of the New York Academy of Sciences* **1249**:211–226.

LaPointe DA, Goff ML, Atkinson CT. 2010. Thermal Constraints to the Sporogonic Development and Altitudinal Distribution of Avian Malaria Plasmodium relictum in Hawai’i. *Journal of Parasitology* **96**:318–324.

Liao W, Elison Timm O, Zhang C, Atkinson CT, LaPointe DA, Samuel MD. 2015. Will a warmer and wetter future cause extinction of native Hawaiian forest birds? *Global Change Biology* **21**:4342–4352.

Maly K. 2004. He moʻolelo ʻāina: a cultural study of the Puʻu Makaʻala Natural Area Reserve districts of Hilo and Puna, island of Hawaiʻi. Available from https://ulukau.org/ulukau-books/?a=d&d=EBOOK-MAKAALA.2.1.1&e=-------haw-20--1--txt-txPT----------- (accessed August 21, 2023).

Maly K, Maly O. 2005. He wahi moʻolelo no Keauhou a me na wahi pana ma laila: a collection of traditions, historical accounts and kamaʻāina recollections of Keauhou and its storied places — Ulukau books. Kumu Pono Associates LLC, Hilo, Hawaiʻi. Available from https://ulukau.org/ulukau-books/?a=d&d=EBOOK-HIKEAU.2.5.11&e=-------haw-20--1--txt-txPT----------- (accessed March 28, 2024).

Messina S, Edwards DP, AbdElgawad H, Beemster GTS, Tomassi S, Benedick S, Eens M, Costantini D. 2020. Impacts of selective logging on the oxidative status of tropical understorey birds. *Journal of Animal Ecology* **89**:2222–2234.

Messina S, Edwards DP, Van Houtte N, Tomassi S, Benedick S, Eens M, Costantini D. 2022. Impacts of selective logging on haemosporidian infection and physiological correlates in tropical birds. *International Journal for Parasitology* **52**:87–96.

Names GR, Schultz EM, Hahn TP, Hunt KE, Angelier F, Ribout C, Klasing KC. 2022. Variation in immunity and health in response to introduced avian malaria in an endemic Hawaiian songbird. *Animal Conservation* **25**:455–466.

Navine AK, Paxton KL, Paxton EH, Hart PJ, Foster JT, McInerney N, Fleischer RC, Videvall E. 2022. Microbiomes associated with avian malaria survival differ between susceptible Hawaiian honeycreepers and sympatric malaria-resistant introduced birds. *Molecular Ecology*:1–12.

Nilsson J-Å, Råberg L. 2001. The resting metabolic cost of egg laying and nestling feeding in great tits. *Oecologia* **128**:187–192.

Nord A, Williams JB. 2015. The enegetic costs of incubation. Page in Deeming DC, Reynolds SJ, editors. Nests, Eggs, and Incubation: New Ideas about Avian Reproduction. Oxford University Press.

Owen JC, Sogge MK, Kern MD. 2005. Habitat and Sex Differences in Physiological Condition of Breeding Southwestern Willow Flycatchers (Empidonax Trailliiextimus). *The Auk* **122**:1261–1270.

Paxton EH, Camp RJ, Gorresen PM, Crampton LH, LeonardJr DL, VanderWerf EA. 2016a. Collapsing avian community on a Hawaiian island. *Science Advances*DOI: 10.1126/sciadv.1600029. American Association for the Advancement of Science. Available from https://www.science.org/doi/abs/10.1126/sciadv.1600029 (accessed January 30, 2022).

Paxton EH, McLaughlin R, Levins S, VanderWerf E, Lancaster N. 2016b. Aging and sexing guide to the forest birds of Hawaiʻi Island. Page 77 Aging and sexing guide to the forest birds of Hawaiʻi Island. Other Government Series 079, HCSU Technical Report Series. University of Hawai'i at Hilo. Available from http://pubs.er.usgs.gov/publication/70179208 (accessed February 2, 2022).

Paxton KL, Cassin-Sackett L, Atkinson CT, Videvall E, Campana MG, Fleischer RC. 2023. Gene expression reveals immune response strategies of naïve Hawaiian honeycreepers experimentally infected with introduced avian malaria. *Journal of Heredity* **114**:326–340.

Pérez-Rodríguez L, Romero-Haro AA, Sternalski A, Muriel J, Mougeot F, Gil D, Alonso-Alvarez C. 2015a. Measuring Oxidative Stress: The Confounding Effect of Lipid Concentration in Measures of Lipid Peroxidation. *Physiological and Biochemical Zoology* **88**:345–351. The University of Chicago Press.

Pérez-Rodríguez L, Romero-Haro AA, Sternalski A, Muriel J, Mougeot F, Gil D, Alonso-Alvarez C. 2015b. Measuring Oxidative Stress: The Confounding Effect of Lipid Concentration in Measures of Lipid Peroxidation. *Physiological & Biochemical Zoology* **88**:345–351.

Pukui MK. 1983. ʻŌlelo Noʻeau: Hawaiian proverbs & poetical sayings. Bishop Museum Press, Honolulu, HI.

Purwaningrum M, Nugroho HA, Asvan M, Karyanti K, Alviyanto B, Kusuma R, Haryanto A. 2019. Molecular techniques for sex identification of captive birds. *Veterinary World* **12**:1506–1513.

Risely A, Klaassen M, Hoye BJ. 2018. Migratory animals feel the cost of getting sick: A meta-analysis across species. *Journal of Animal Ecology* **87**:301–314.

Rodríguez P, Tortosa FS, Gortázar C. 2006. Daily variations of blood biochemical parameters in the red-legged partridge (Alectoris rufa). *European Journal of Wildlife Research* **52**:277–281.

Samuel MD, Liao W, Atkinson CT, LaPointe DA. 2020. Facilitated adaptation for conservation – Can gene editing save Hawaii's endangered birds from climate driven avian malaria? *Biological Conservation* **241**:108390.

Samuel MD, Woodworth BL, Atkinson CT, Hart PJ, LaPointe DA. 2015. Avian malaria in Hawaiian forest birds: infection and population impacts across species and elevations. *Ecosphere* **6**:art104.

Schoener E, Banda M, Howe L, Castro I, Alley M. 2014. Avian malaria in New Zealand. *New Zealand Veterinary Journal* **62**:189–198. Taylor & Francis.

Schoenle LA, Kernbach M, Haussmann MF, Bonier F, Moore IT. 2017. An experimental test of the physiological consequences of avian malaria infection. *Journal of Animal Ecology* **86**:1483–1496.

Sepp T, Karu U, Blount JD, Sild E, Männiste M, Hõrak P. 2012. Coccidian Infection Causes Oxidative Damage in Greenfinches. *PLOS ONE* **7**:e36495.

Smetzer JR, Paxton KL, Hart PJ, Paxton EH. 2022. Activity patterns of Hawaiian forest birds in a fragmented and continuous landscape. *Journal of Avian Biology*.

van Riper III C, van Riper SG, Goff ML, Laird M. 1986. The Epizootiology and Ecological Significance of Malaria in Hawaiian Land Birds. *Ecological Monographs* **56**:327–344.

Vézina F, Williams TD. 2003. Plasticity in Body Composition in Breeding Birds: What Drives the Metabolic Costs of Egg Production? *Physiological and Biochemical Zoology* **76**:716–730. The University of Chicago Press.

Videvall E, Paxton KL, Campana MG, Cassin-Sackett L, Atkinson CT, Fleischer RC. 2021. Transcriptome assembly and differential gene expression of the invasive avian malaria parasite Plasmodium relictum in Hawaiʻi. *Ecology and Evolution* **11**:4935–4944.

Villegas A, Guzman J, Corbacho Amado C, Corbacho P, Vargas J. 2004. Blood values of Bald Ibis (Geronticus eremita) in captivity: Comparative ranges and variability with age, sex and physical condition. *Journal of Ornithology* **145**:98–104.

Wagner EC, Prevolsek JS, Wynne-Edwards KE, Williams TD. 2008. Hematological changes associated with egg production: estrogen dependence and repeatability. *Journal of Experimental Biology* **211**:400–408.

Yorinks N, Atkinson CT. 2000. Effects of malaria on activity budgets of experimentally infected juvenile Apapane (Himatione sanguinea). *The Auk* **117**:731–738.

Zehtindjiev P, Ilieva M, Westerdahl H, Hansson B, Valkiūnas G, Bensch S. 2008. Dynamics of parasitemia of malaria parasites in a naturally and experimentally infected migratory songbird, the great reed warbler Acrocephalus arundinaceus. *Experimental Parasitology* **119**:99–110.