



Technical Report HCSU-042

IMMUNOLOGICAL MARKERS FOR TOLERANCE TO AVIAN
MALARIA IN HAWAI`I `AMAKIHI: NEW TOOLS FOR
RESTORING NATIVE HAWAIIAN FOREST BIRDS?

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August 2013



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This product was prepared under Cooperative Agreement CAG11AC20269 for the Pacific Island Ecosystems Research Center of the U.S. Geological Survey.



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NONTECHNICAL SUMMARY

Expansion of deadly, mosquito-borne bird diseases such as avian malaria into Hawai'i's high elevation forests as a result of global warming is one of the most significant threats facing the state's rare native forest birds. Few practical options for control of disease-carrying mosquitoes over large landscapes are available, however. The best hope for remaining species of native birds may be the development of tolerance or resistance to this introduced disease. Therefore, understanding how tolerance and resistance evolve and developing ways to speed this process may be good proactive strategies for addressing impacts of climate change. We evaluated ways to rapidly measure a bird's natural immunity with the hope that they can predict survival from avian malaria. We evaluated cell-mediated immune responses, measured levels of natural antibodies (NAb) in the blood plasma, and measured plasma antioxidant capacity with the hypothesis that these tests may be able to distinguish disease-tolerant low-elevation populations of Hawai'i 'Amakihi (*Hemignathus virens*) from disease-susceptible high-elevation populations. We found no significant differences in amount of wing web swelling associated with cellular immune responses and no significant differences in plasma antioxidant capacity between low- and high-elevation 'Amakihi. We observed significant differences in levels of NAb from high- and low-elevation birds in an assay that measured complement-mediated lysis of rabbit erythrocytes. This assay is rapid, not affected by malarial infection status, relatively inexpensive, and works for a wide range of species, making it particularly attractive as a management tool for distinguishing malaria-tolerant and susceptible individuals. Additional work is needed to determine whether high levels of NAb correlate with ability to recover from avian malaria in 'Amakihi and other native honeycreepers and whether this assay can be used to map geographic populations of native forest birds that are tolerant of this disease.

ABSTRACT

We evaluated three assays for non-specific or innate immune capacity to see if measurements were independent of malarial infection and capable of distinguishing malaria-tolerant, low-elevation Hawai'i 'Amakihi from malaria-susceptible, high-elevation 'Amakihi. 'Amakihi were captured at Malama Ki Forest Reserve (20 m), Hakalau Forest National Wildlife Refuge (1800 m), and Upper Waiakea Forest Reserve (1700 m), bled for collection of plasma and packed erythrocytes for malarial diagnostics, and either transported to Kīlauea Field Station Aviary and held in captivity for 48 hours for inoculation of wing webs with phytohemagglutinin A (PHA) or released immediately in the field after collection of a blood sample. All birds were tested by polymerase chain reaction (PCR) and microscopy to determine infection status. We found no significant association between malarial infection status and degree of wing web swelling after inoculation with PHA ($T = -0.174$, $df = 13$, $P = 0.864$) and no association between origin of birds from low- and high-elevation populations and degree of wing web swelling ($T = 0.113$, $df = 52$, $P = 0.911$). Infected 'Amakihi from low elevation had significantly higher small molecule plasma antioxidant capacity than uninfected individuals from the same population ($T = -2.675$, $df = 21$, $P = 0.014$), so we limited comparisons to uninfected birds. Uninfected 'Amakihi from low elevations did not differ in small molecule plasma antioxidant capacity from uninfected 'Amakihi from high elevation ($T = -0.260$, $df = 46$, $P = 0.796$). Compared to high-elevation birds, low-elevation 'Amakihi had significantly higher titers of natural antibodies (NAb) as measured by complement-mediated lysis of rabbit erythrocytes (Mann-Whitney $U = 27$, $\chi^2 = 32.332$, $df = 1$, $P < 0.0001$). This innate immunological difference may be related to ability to

survive malarial infection and may prove to be important for understanding possible mechanisms for the evolution of disease tolerance in Hawai'i's native bird species.

INTRODUCTION

Introduced avian malaria and pox virus continue to play a major role in limiting the altitudinal and geographic distribution of native Hawaiian birds (Atkinson and LaPointe 2009). The persistence and dynamics of these mosquito-transmitted diseases are influenced by vector and host abundance, climatic components (temperature and rainfall) that drive the system, and landscape components (elevation and mosquito habitat) that influence rates of transmission and resulting selective pressures on forest bird populations (Samuel *et al.* 2011). Climate strongly influences mosquito biology because temperature affects larvae maturation, female blood feeding and reproductive (gonadotropic) cycle, and parasite development within the mosquito. Rainfall also affects mosquito survival because drought or strong rain can decrease the survival of larvae and adults (Ahumada *et al.* 2004, Samuel *et al.* 2011). Due to the steep slopes of many Hawaiian Islands, climate, especially temperature, changes quickly across elevations, creating strong differences in system dynamics that have resulted in relatively stable persistence of high-elevation disease refugia on the highest mountains of Kaua'i, Maui, and Hawai'i Islands. These refugia have allowed many species of endemic forest birds that are highly susceptible to avian malaria based on laboratory experiments (e.g., 'I'iwi; Atkinson *et al.* 1995) to persist at high elevations. The long-term stability of these high-elevation refugia is increasingly threatened by changing climatic conditions in the islands, particularly the recent upward trends in night-time temperatures at high elevations and stable or downward trends in the upper limits of the inversion layer, which currently caps forest bird habitat at approximately 2000 m (Giambelluca *et al.* 2008, Diaz *et al.* 2011). There is increasing evidence that disease transmission is already moving into high-elevation habitats and having an impact on susceptible native forest birds. For example, prevalence of avian malaria has tripled over the past decade at the lower end of the Alaka'i Plateau and increased almost 10-fold near its upper reaches (Atkinson and Utzurrum 2010). During the same time period, the range of native forest birds in the Alaka'i has contracted substantially in size around the highest elevations on the island (Foster *et al.* 2004).

Few proven methods exist for countering the threat of climate change on disease exposure in native forest birds. While vector control is a feasible option for reducing transmission of avian malaria in many parts of Hawai'i, it may not be sustainable over the long haul because of the expense and scale of effort that are needed over large, contiguous landscapes (LaPointe *et al.* 2009). Some mid- and high-elevation habitats have little natural standing water and are excellent candidates for the development of effective vector control programs, but other areas of critical forest bird habitat have abundant natural water sources and available larval habitat for mosquitoes or are too small in area or too close to parcels where vector control will never be a feasible priority. Additionally, establishing forest habitat in successively higher elevation preserves to keep forest birds "one step ahead" of rising temperatures may be an effective short-term solution in some places, but eventually temperatures will rise to where even higher elevation habitat does not provide refuge from disease. Development of disease tolerance may ultimately be required for long-term sustainability of all of Hawai'i's forest birds.

The recent finding that some low-elevation populations of Hawai'i 'Amakihi are recovering and expanding in spite of high rates of malaria transmission (Woodworth *et al.* 2005, Spiegel *et al.*

2006) has opened the possibility that at least some native forest birds have sufficient genetic diversity to evolve some tolerance to avian malaria. Genetic differences have been detected between low- and high-elevation populations of Hawai'i 'Amakihi with microsatellites (Eggert *et al.* 2008) and nuclear and mitochondrial markers (Foster *et al.* 2007), but these differences appear to be based on a variety of haplotypes and no specific genetic markers have yet been identified that can accurately and quickly predict susceptibility to infection. Complicating this is the likelihood that tolerance to malaria most likely involves multiple genes or gene systems (Hedrick 2011). Reliable genetic markers for identifying disease-tolerant individuals are currently not available.

An alternative approach to identifying tolerant or resistant individuals is to evaluate phenotypic characteristics associated with the immune response in the hopes that one or more of these may predict ability to recover from malarial infection. If we can find one or more useful phenotypic markers, this approach could be a "game changer" in how we manage native forest birds in a warming climate. For example, this approach could be used to identify priority populations with high levels of natural disease tolerance for intensive management, target individual birds for use in translocations to lower elevations where disease transmission is endemic, identify offspring from captive propagation programs for release in areas with disease transmission, or establish that some populations may not have the genetic and phenotypic variability to adapt over the long run to increased disease transmission at higher elevations.

The goal of our study was to evaluate three assays that measure non-specific innate immunity in low- and high-elevation Hawai'i 'Amakihi with the eventual hope that one or more may predict susceptibility to avian malaria.

METHODS

Sample Collection

Hawai'i 'Amakihi were captured with mist nets from March–November 2012 on the island of Hawai'i at Malama Ki Forest Reserve near MacKenzie State Park (20 m), at the Pua Akala Management Unit of Hakalau Forest National Wildlife Refuge (1800 m), and at several kīpuka along Powerline Road in the Upper Waiakea Forest Reserve (1700 m). After capture, birds were banded with U.S. Geological Survey aluminum leg bands, measured to obtain morphometric data for identifying sex and age, weighed, and either bled immediately by jugular venipuncture with a heparinized 28 gauge insulin syringe or bled after transport to Kīlauea Field Station Aviary in Hawai'i Volcanoes National Park. A blood smear was prepared immediately after blood was drawn, air dried, and fixed with methanol. Remaining whole blood was transferred to a microhematocrit tube and spun with either a battery-powered field centrifuge or a laboratory hematocrit centrifuge to separate plasma from packed cells. Microhematocrit tubes were scored with a file, broken immediately above the buffy coat, and plasma and packed cells were removed. Plasma was transferred to 0.5 ml tubes, stored on wet ice until return to the lab, and then frozen at -70°C for long-term storage. Packed cells were placed in 100 µl of lysis buffer (0.1 M Tris, pH 8.0, 0.1 M EDTA, 2% SDS), stored on wet ice until return to the lab, and then frozen at -70°C for long-term storage.

All samples were collected under terms of State of Hawai'i Protected Wildlife Permit WL13-07, Federal Migratory Bird Permit MB45431A-0, Hakalau Forest National Wildlife Refuge Special Use Permit SUP12516-12015, and University of Hawai'i Animal Care and Use Protocol 11-1170.

Diagnostic Analysis for Malaria

Since acute or chronic infection with malaria could bias test results, we screened all birds by microscopy and polymerase chain reaction (PCR) assays to establish infection status for each bird. Blood smears were stained with phosphate buffered (pH 7.0) 2% Giemsa for one hour, rinsed with tap water, dried, and examined by microscopy to detect intraerythrocytic stages of *Plasmodium*. We screened each smear for 10 min at 400X (40X objective and 10X eyepieces) and estimated that we examined approximately 20,000–30,000 erythrocytes. Smears were scored as positive for malaria if we observed at least one infected erythrocyte.

Purified DNA for PCR analysis was extracted from packed blood cells using DNeasy tissue extraction kits (Qiagen Inc., Valencia, CA) according to the manufacturer's protocols, but we increased the initial incubation times with Proteinase K to overnight to increase yield of DNA. DNA was recovered from extraction columns with Tris ethanolamine buffer, measured by spectrophotometry with a Nanodrop spectrophotometer to assess purity and determine DNA concentration, and stored frozen until use in PCR reactions.

We used published PCR primers that amplify parasite ribosomal genes for detecting infection with *Plasmodium* (Fallon *et al.* 2003). The primers were used in a nested protocol with an initial amplification of host DNA (100 ng/reaction) with primers 292F/631R followed by a second amplification with primers 343F/496R that used 1 µl of a 1:10 dilution of template from the first reaction. PCR reactions with primers 292F/631R were run in 25 µl volumes containing the following components in the reaction mix: 2.0 mM MgCl₂, 0.2 mM each deoxynucleoside triphosphate, 0.4 µM each primer, and 0.5 units of Promega GoTaq polymerase (Promega North America, Madison, WI). PCR reactions with primers 343F/496R were run in 25 µl volumes containing the following components in the reaction mix: 2.5 mM MgCl₂, 0.2 mM each deoxynucleoside triphosphate, 0.5 µM each primer, and 0.25 units of Promega GoTaq polymerase. Cycling conditions for the original flanking primer pair (292F/631R) followed a hot-start, touch-down protocol: 2 min at 94°C, followed by 20 cycles with 1-min denaturation at 94°C, 1-min annealing at 52°–42°C, and elongation at 72°C for 1 min and 10 sec. After 20 cycles, a final elongation step followed at 72°C for 3 min. The final assay primer pair (343F/496R) was run at 2 min at 94°C, followed by 35 cycles with 1-min denaturation at 94°C, 1-min annealing at 57°C, and elongation at 72°C for 1 min and 10 sec, with a final elongation step at 72°C for 3 min. PCR products from the second reaction were resolved on 1.5% agarose gels to determine presence or absence of an expected 142 bp band. All PCR reactions were run with a positive control consisting of DNA extracted from a Pekin duckling with an intense experimental infection with *P. relictum* and a negative control that substituted water for DNA.

Wing Web Inoculation

Amakihi were held in the field in small transport cages, offered fresh fruit and nectar substitute (Nekton Nectar Plus, Gunter Enderle, Clearwater, FL) and transported within five hours after capture to either Kīlauea Field Station Aviary or the University of Hawai'i Hakalau Field Station. Birds that arrived at the aviary or field station before 1400 were inoculated in the left wing web with 20 µl of a 5 mg/ml solution of phytohemagglutinin A (Catalog #L 9017, Sigma-Aldrich Chemical Company, St. Louis, MO) that had been reconstituted from a lyophilized state with sterile, phosphate buffered saline (PBS), pH 7.4, without added calcium chloride or magnesium chloride (Catalog #10010-023, GIBCO Life Technologies, Grand Island, NY; Martin *et al.* 2006). The right wing web was inoculated immediately afterward with 20 µl of sterile PBS alone. Immediately prior to inoculation, the thickness of both the left and right wing webs was measured with a digital spessimeter (Mitutoyo No. 547-300, Mitutoyo Corporation America,

Aurora, IL) with the wing gently opened to an outstretched position. Birds were held overnight in cages measuring 60 X 30 X 30 cm with a 500 watt heat lamp positioned above one corner of the cage to provide an external heat source. Birds were offered Nekton Nectar Plus, scrambled eggs, frozen blueberries, frozen kernel corn, and an orange slice *ad libitum*. Twenty-four hours after the wing web inoculations, left (treatment) and right (control) wing webs were measured a second time with a spessimeter to record changes in thickness. All pre- and post-injection measurements were done by a single individual (CTA) to minimize observer variability in the measurements. Birds were held an additional night to avoid release late in the afternoon and set free at their capture location.

Total Antioxidant Capacity Assay

Total antioxidant capacity (TAC) was measured for plasma samples using a BioVision Total Antioxidant Capacity Assay Kit (BioVision Incorporated, Milpitas, CA). The assay is based on reduction of Cu^{2+} to Cu^+ by antioxidant enzyme systems such as catalase, peroxidase, and glutathione reductase; small molecules such as ascorbate, uric acid, and glutathione; and proteins such as albumin and transferrin. Reduced Cu^+ is chelated with a colorimetric probe which leads to a color change and broad absorbance peak around 570 nm that is proportional to the TAC. A standard curve is prepared with Trolox, a water-soluble derivative of vitamin E, and antioxidant capacity for each sample is expressed in Trolox equivalents. We chose to use a proprietary protein mask that was supplied with the kit which allows analysis of only the small molecule antioxidants in the plasma samples, after initial tests without the mask did not detect any differences between low- and high-elevation 'Amakihi.

We performed the assay by mixing 1 μl of plasma with 1 μl of protein mask and diluting the mixture 1:10 with water. We added 4 μl of the dilution to duplicate wells (two per sample) of a flat-bottomed 96-well microtiter plate (Immulon 4HBX, #3855, ThermoFisher Scientific, Milford, MA) and adjusted the volume of all wells to 100 μl by adding 96 μl of distilled water to each well. We also prepared a series of Trolox standards by adding 0, 4, 8, 12, 16, and 20 μl of Trolox to six wells and adjusting the volume of each to 100 μl with distilled water. Two wells were used as blanks and contained 100 μl of distilled water alone. We added 100 μl of Cu reagent to all wells and incubated for 90 min at room temperature. Absorbance was read at 595 nm with a BioRad plate reader (BioRad Life Sciences, Hercules, CA), and average values in Trolox equivalents were calculated for all samples from the standard curve by averaging each of the duplicate samples.

Natural Antibody Assay

We followed the hemolysis-hemagglutination assay described by Matson *et al.* (2005) and modified by Zylberberg *et al.* (2012) to use smaller quantities of plasma to compare titers of NAb in low- and high-elevation 'Amakihi. The assay was done in 96-well (8 rows by 12 columns) round (U) bottom assay plates (Corning Costar #3795, Tewksbury, MA). Ten microliters of plasma from each bird was pipetted into wells of column 1 of the plate (six individual samples). The remaining two wells in column 1 were used as positive controls to evaluate plate-to-plate variability and contained 10 μl of pooled plasma from three chickens. Ten microliters of 0.01 M PBS (Sigma #P3813, St Louis, MO) was added to all wells in columns 1–12 with a multichannel pipetter. The contents of wells in column 1 were then mixed simultaneously with a multi-channel pipetter and 10 μl were removed from wells in column 1 and transferred to adjacent wells in column 2. The contents of wells in column 2 were mixed and 10 μl were removed and transferred to wells in column 3. This process was repeated for wells 3–11 to yield a series of

dilutions ranging from 1/2 (column 1) to 1:2048 (column 11). The last aliquot from column 11 was discarded, leaving only PBS in wells of column 12 to serve as negative controls.

For the assay itself, 10 μ l of a 1% rabbit blood cell were added to all wells, effectively halving all plasma dilutions. The 1% solution was prepared from a 50% suspension of rabbit whole blood in Alsever's solution (Hemostat Laboratories, # RB*050 in Alsever's solution, Dixon, CA) as described in detail by Matson *et al.* (2005). Each plate was then sealed with clear adhesive plate film to prevent evaporation. Plates were gently mixed on a rotating shaker for 30 sec and then floated in a 37°C water bath for 90 min. Upon completion of the incubation, the long axis of each plate was tilted to a 45° angle for 20 min at room temperature in order to enhance visualization of agglutination. Plates were then placed on a light box and photographed. Afterward, plates were incubated flat at room temperature for an additional 70 min and photographed a second time to record maximum lytic activity (Figure 1). From the digitized images, both agglutination and lysis were scored independently and blindly by two observers (EHP and CTA), and values were averaged for data analysis. Lysis reflects the interaction of complement and NAb, whereas agglutination results from NAb only. Both variables were recorded as the negative log₂ of the last plasma dilution exhibiting each behavior as described in detail by (Matson *et al.* 2005).

Statistical Analysis

We compared mean values for changes in wing web thickness, NAb titer, and antioxidant capacity for infected and uninfected low-elevation 'Amakihi with two-sample t-tests, $P < 0.05$, to determine if values were independent of malarial infection. When no significant differences were evident, we tested for differences between low- and high-elevation 'Amakihi with a two-sample t-test, $P < 0.05$. When data were skewed significantly by the frequent occurrence of zero values, we evaluated differences with a Mann-Whitney test, the non-parametric equivalent of a two-sample t-test. All statistical tests were done using the statistical package Systat (version 11, Systat Software, Inc., Chicago, IL).

RESULTS

Sample Collection and Malarial Diagnostics

We captured and collected plasma and packed red blood cells from 60 Hawai'i 'Amakihi from Upper Waiakea Forest Reserve (37 birds), Malama Ki Forest Reserve (18 birds), and Hakalau Forest National Wildlife Refuge (5 birds) between February and October 2012. We were able to perform successful wing web inoculations on 54 of these birds.

The prevalence of malaria by PCR analysis from birds that were captured at Malama Ki Forest reserve was 61% (11/18). Among 42 'Amakihi captured at high elevation (Hakalau Forest National Wildlife Refuge and Upper Waiakea Forest Reserve), only one (2.3%) was positive for malaria. By contrast, prevalence as detected by microscopy was 39% at Malama Ki and 0% among high-elevation birds.

Wing Web Inoculation

We compared pre- and post-wing web thickness for 50 birds that were successfully inoculated with PBS to evaluate whether inoculation with PBS alone caused a significant change in thickness after 24 hours. Mean pre-injection wing web thickness ($\bar{X} = 0.420$ mm) did not differ significantly from mean post-injection wing web thickness ($\bar{X} = 0.434$ mm; $t = -1.664$, $df = 49$,

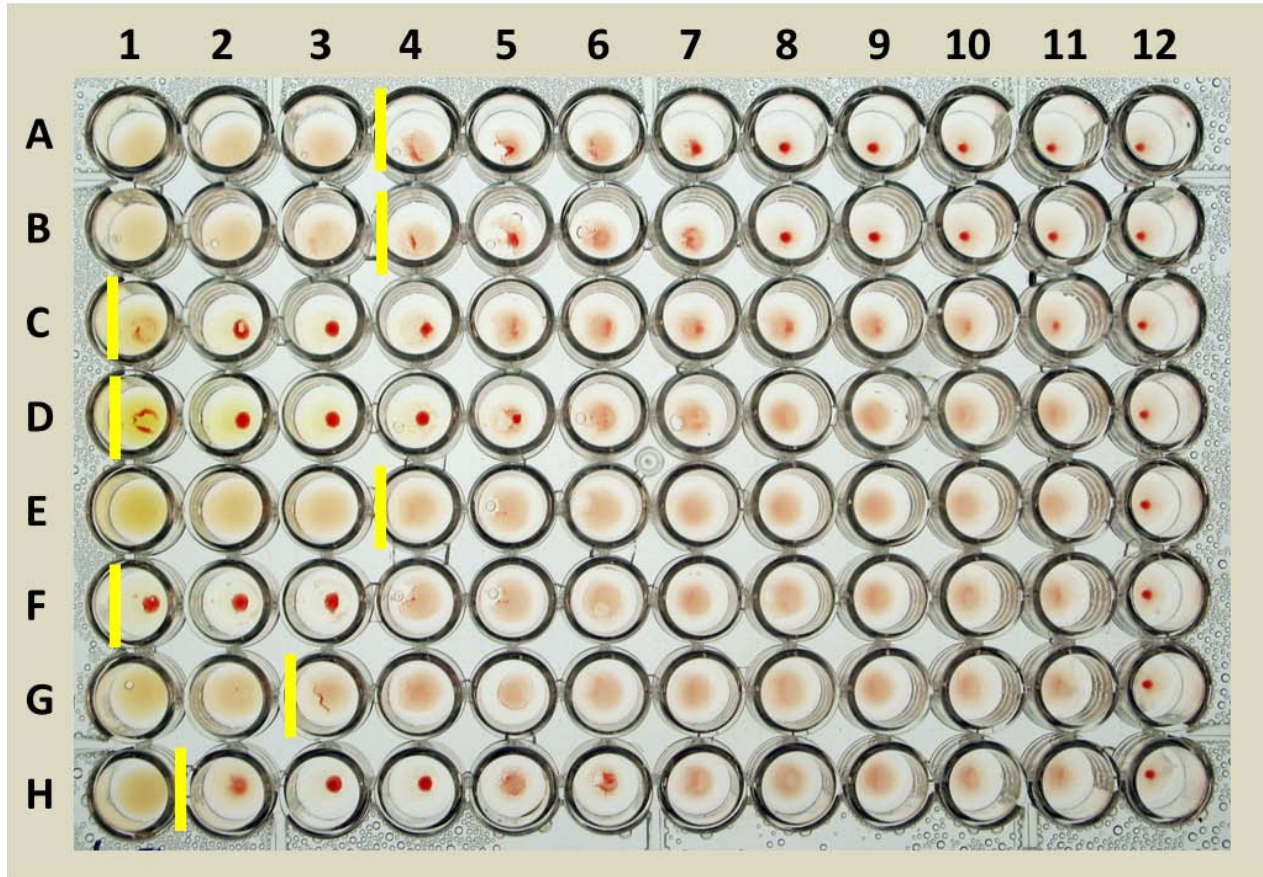


Figure 1. Representative assay plate that illustrates natural antibodies (Nab) and complement-mediated lysis of rabbit red blood cells after 180 minutes of incubation. Columns 1–11 contain rabbit red blood cells and serially two-fold diluted plasma ranging in titer from 1:2 (column 1) to 1:2048 (column 11). Column 12 (control) contains phosphate buffered saline and rabbit cells alone. Rows A and B contain pooled chicken plasma to control for plate-to-plate variability of results. Rows C–H contain plasma from individual 'Amakihi that originated from either low- (Rows E, G) or high- (Rows C, D, F, H) elevation habitats. Approximate cut-off values, based on partial or complete lysis of rabbit red blood cells, are indicated for each row with a yellow bar. Note the higher lysis scores for low-elevation 'Amakihi in Rows E and G.

$P = 0.103$). As a result, unadjusted changes in wing web thickness (DeltaWing) were used for comparisons of infected and uninfected individuals and high- and low-elevation individuals.

Among low-elevation 'Amakihi, we found no significant difference in mean DeltaWing for low-elevation birds with ($\bar{X} = 0.305$, $SD = 0.120$, $n = 8$) or without ($\bar{X} = 0.296$, $SD = 0.079$, $n = 7$) ($t = -0.174$, $df = 13$, $P = 0.864$) malaria. Since this difference was not significant, we pooled infected and uninfected birds from low elevation for comparisons with high-elevation birds to increase sample sizes. There was no significant difference in mean DeltaWing between low- ($\bar{X} = 0.301$, $SD = 0.099$, $n = 15$) and high- ($\bar{X} = 0.304$, $SD = 0.111$, $n = 39$) elevation 'Amakihi ($t = 0.113$, $df = 52$, $P = 0.911$).

Total Antioxidant Capacity Assay

Low-elevation 'Amakihi that tested positive for malaria had significantly higher measurements of small molecule plasma antioxidant capacity ($\bar{X} = 15.318$, $SD = 2.742$, $n = 16$) than those that were uninfected ($\bar{X} = 12.185$, $SD = 2.140$, $n = 7$; $t = -2.675$, $df = 21$, $P = 0.014$). Because of the potential confounding effects of malaria infection on comparisons between low- and high-elevation birds, we limited comparisons to uninfected birds in each group. There were no significant differences in small molecule plasma antioxidant capacity between uninfected low- ($\bar{X} = 12.185$, $SD = 2.140$, $n = 7$) and high- ($\bar{X} = 11.997$, $SD = 1.715$, $n = 41$) elevation 'Amakihi ($t = -0.260$, $df = 46$, $P = 0.796$).

Natural Antibody Assay

While control chicken plasma yielded a consistent agglutination endpoint at plasma dilutions of 1:128, we did not find an agglutination endpoint for any of the 'Amakihi samples. A flocculent precipitate was present at the last plasma dilution (1:2048) for virtually all of the birds, and this variable received no further analysis.

We found little plate-to-plate variability for the pooled chicken plasma control, so we used unadjusted scores for the analysis. Lysis scores of plasma from uninfected ($\bar{X} = 2.644$, $SD = 1.056$, $n = 7$) and infected ($\bar{X} = 2.558$, $SD = 1.064$, $n = 11$) low-elevation 'Amakihi did not differ significantly ($t = 0.168$, $df = 16$, $P = 0.869$), so infected and uninfected low-elevation birds were combined for comparison with high-elevation birds. We used a non-parametric Mann-Whitney test to evaluate differences between high- and low-elevation 'Amakihi because high-elevation data were significantly skewed by large numbers of zero scores (Figure 2). Low-elevation 'Amakihi had significantly higher lysis scores than high-elevation birds (Mann-Whitney $U = 27$, $X^2 = 32.332$, $df = 1$, $P < 0.0001$), indicating that they have higher titers of NAb.

DISCUSSION

The idea that native forest birds might develop resistance or tolerance to avian malaria was first discussed by van Riper *et al.* (1986) based on higher rates of survival in a limited number of experimentally infected Hawai'i 'Amakihi that originated from different elevations on Mauna Loa Volcano. Described as "immunogenetic" resistance, this concept has been an attractive option for limiting the impacts of this introduced disease on native forest birds for more than two decades (U.S. Fish and Wildlife Service 2006). The first clear evidence that natural selection can lead to adaptation to this disease was the discovery of a rapidly expanding population of Hawai'i 'Amakihi in the lower Puna District of Hawai'i Island in spite of high rates of infection with malaria (Woodworth *et al.* 2005, Spiegel *et al.* 2006). Experimentally infected birds from this population exhibit greater tolerance to infection. Mortality is lower, and declines in food consumption and weight loss are significantly less than high-elevation birds in spite of similar intensities of infection (C.T. Atkinson unpublished data). Genetic differences between these low-elevation 'Amakihi and high-elevation birds have been documented based on nuclear and mitochondrial markers (Foster *et al.* 2007, Eggert *et al.* 2008), yet the mechanisms responsible for evolved tolerance are not known, and unique genetic markers that could be used to identify disease-tolerant individuals are not currently available. While recent developments in whole genome sequencing show great promise for identifying single nucleotide polymorphisms that may be associated with disease susceptibility, they may not be available for practical application for some time.

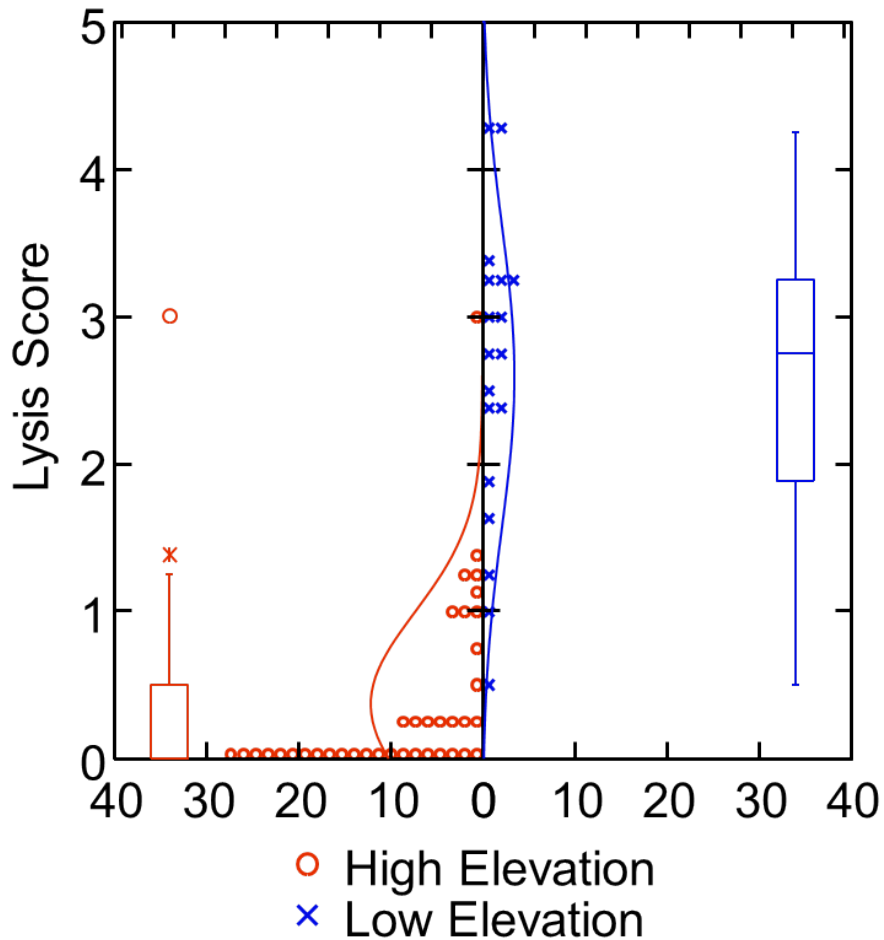


Figure 2. Plot of lysis scores (Y axis) from the assay for natural antibodies (NAb). X axis is frequency distribution (counts) of lysis scores for high- (left) and low- (right) elevation birds. Red and blue curves are smoothing splines for the frequency distributions. Box plots on either side of the curves show median (line) within a box that defines the first and third quartiles of the data. Whiskers represent minimum or maximum values. High-elevation 'Amakihi had lower levels of NAb as measured by cell lysis ($\bar{X} = 0.369$, $SD = 0.624$, $n = 38$) than low-elevation 'Amakihi ($\bar{X} = 2.592$, $SD = 1.030$, $n = 18$). Because of the skewed distribution of scores for high-elevation 'Amakihi, comparison of mean values was done with a non-parametric Mann-Whitney Test. Differences were highly significant ($P < 0.0001$, $U = 27$, $\chi^2 = 32.332$, $df = 1$).

We hypothesized that if genetic differences in susceptibility to malaria exist, measurable phenotypic differences in either immune competence or ability to tolerate malarial infection might differentiate low-elevation disease-tolerant 'Amakihi from high-elevation malaria-susceptible 'Amakihi. We tested this hypothesis by using three assays that are widely used in the field of ecological immunology to measure immune competence and fitness in wild passerines (Salvante 2006, Millet *et al.* 2007). The phytohemagglutinin skin swelling test has been widely used to estimate cell-mediated immunity, particularly T cell-mediated responses, but more recent work has shown that it more accurately reflects the ability to mount an

inflammatory response (Vinkler *et al.* 2010). Since cytokines, chemokines, and reactive oxygen intermediates produced by macrophages in response to acute infection can mediate pathology of malarial infection (Clark and Cowden 2003, Chu *et al.* 2013), we evaluated the hypothesis that disease-tolerant, low-elevation 'Amakihi can control tissue damage through elevated plasma antioxidant capacity. Finally, we evaluated titers of NABs in high- and low-elevation 'Amakihi using a hemolysis-hemagglutination assay that was first described by Matson *et al.* (2005). Natural antibodies (also referred to as spontaneous antibodies) are part of the innate immune response and are produced without prior exposure to specific antigens in immunologically naïve vertebrates (Ochsenbein and Zinkernagel 2000). Their role in the immune response is still poorly understood, but they are believed to have immunoregulatory functions and play an important role as a first line of defense against bacteria and viruses by binding and lysing pathogens in conjunction with complement, enhancing phagocytosis by macrophages, and targeting pathogens to the spleen and other lymphoid tissue where they can prime the adaptive immune response (Ochsenbein and Zinkernagel 2000). The NAb response is under genetic control (Ochsenbein and Zinkernagel 2000, Wijga *et al.* 2009) and may predict both the strength of the adaptive immune response (Kohler *et al.* 2003, Parmentier *et al.* 2004) and host survival (Sun *et al.* 2011).

We did not find significant differences between low- and high-elevation 'Amakihi for wing web swelling after inoculation of phytohemagglutinin or small molecule plasma antioxidant capacity. This was somewhat surprising given the role that the inflammatory response plays in the pathogenesis of malaria (Clark and Cowden 2003), but it may be that these effects require specific stimulus of the immune system by either malaria infection or malarial antigens to be detectable (Owen *et al.* 2010). Low-elevation infected 'Amakihi had higher levels of small molecule plasma antioxidant capacity than uninfected birds from the same population, suggesting that either infection with malaria is necessary for this phenotypic trait to be expressed or selective pressure from malaria infection may have biased captures of wild birds toward those with higher antioxidant capacity. Additional work that focuses on responses to either crude or purified malarial antigens may be a better approach to detecting differences in cellular immune responses between these populations.

We were able to detect significantly higher titers of NAB in low-elevation 'Amakihi with an assay that measures complement-mediated lysis of rabbit erythrocytes. The results were independent of infection status with malaria, making it particularly useful for screening birds from areas where malaria transmission is seasonal. While it has been hypothesized that increased titers of NAB might correlate with strength of the adaptive immune response (Kohler *et al.* 2003, Parmentier *et al.* 2004) and survival in chickens (Sun *et al.* 2011) empirical evidence for this is still limited. Only a handful of studies have detected specific disease-related responses to measured titers of natural antibodies (Owen *et al.* 2010). Using the same hemolysis-hemagglutination assay that we did, Whiteman *et al.* (2006) detected decreases in ectoparasite loads in birds with higher titers of NAB. Results from other studies have found either no association between ectoparasites and NAB or a positive association (Owen *et al.* 2010), but methodologies varied widely and direct comparisons may not be valid. To date, there have been no studies documenting an association between higher titers of NAB and survival from malaria. While beyond the scope of the current study, the next step would be to see if any detected differences correlate with ability to survive acute infection with malaria. Any assay that is developed, either genetic or otherwise must be rapid, inexpensive, and applicable to a wide range of species to make it useful for the management of forest birds.

CONCLUSIONS AND RECOMMENDATIONS

We show that there are significant differences in mean titers of NAb between high-elevation 'Amakihi populations sensitive to malaria and low-elevation populations that are more tolerant of the disease. This immunological difference may be directly related to their differential survival rates when exposed to disease. The NAb assay we used to detect these differences is rapid, does not require specialized equipment, and can be applied to a wide range of species. The assay may be able to provide information that can be used to select birds for translocation into areas of suitable habitat with documented disease transmission, select offspring from captive propagation programs for release into areas where risks of disease exposure are high, and map the geographic distribution of populations that are tolerant of avian disease. Thus, this study is an important advance in understanding possible mechanisms for the evolution of disease resistance in Hawai'i's native bird species and in understanding how future climate change will affect the distribution and abundance of native forest birds. Additional studies to verify these findings and determine whether NAb titer can predict mortality, survival times, peak parasitemias, or day of crisis in experimentally infected birds would help to determine whether this assay is an accurate predictor of tolerance to avian malaria in 'Amakihi and other species of honeycreepers. It may be possible to do this with frozen plasma samples from prior experimental studies of avian malaria in Hawai'i 'Amakihi, 'Iiwi (*Vestiaria coccinea*), 'Apapane (*Himatione sanguinea*), 'Ōma'ō (*Myadestes obscurus*), and Maui 'Alauahio (*Paroreomyza montana*; Atkinson *et al.* 1995, 2000, 2001; Yorinks and Atkinson 2000) and this should be a high priority next step.

ACKNOWLEDGEMENTS

We thank Keith Burnett, Jackie Gaudio, Aemon Hannity, Nolan Lancaster, and Sonia Levitt for assisting with mist netting and capture of 'Amakihi that were brought into captivity. This project was funded by the Pacific Island Climate Change Cooperative and the U.S. Geological Survey.

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