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CHANGES IN THE PREVALENCE OF AVIAN DISEASE AND MOSQUITO VECTORS AT HAKALAU FOREST NATIONAL WILDLIFE REFUGE: A 14-YEAR PERSPECTIVE AND ASSESSMENT OF FUTURE RISK

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ABSTRACT

Throughout the main Hawaiian Islands, introduced mosquito-borne disease has had, and continues to have, a profound impact on the distributions and abundance of native Hawaiian forest birds. Populations of remaining native forest birds are largely restricted to high elevation forests where mean temperatures are marginal for vector and parasite development and limited availability of larval mosquito habitat constrains mosquito populations and disease transmission. Hakalau Forest National Wildlife Refuge (HFNWR) was established for the preservation of endemic avifauna in 1985. Since its creation, native bird communities there have remained intact and most species populations are stable or increasing. However, avian malaria had been detected at HFNWR in the past and, in light of documented climate change, new concerns have been raised regarding the long-term fate of the refuge's forest birds. To examine the possible changes in avian malaria transmission at HFNWR we sampled forest birds for blood parasites, trapped adult mosquitoes and surveyed larval mosquito habitat at three sites during 2012 and compared our results with similar data collected between 1998 and 1999. We tested blood samples by polymerase chain reaction (PCR), immunoblotting, and microscopy to determine prevalence of acute and chronic infection and used attractive gravid traps to sample the vector mosquito *Culex quinquefasciatus*.

Our study documented spatial trends and temporal changes in the prevalence of avian malaria, mosquito presence, larval mosquito habitat and feral pig activity at HFNWR. We found evidence of local transmission in high elevation forests, a general pattern of increasing prevalence at lower elevations and along a South to North gradient and a two-fold decrease in the prevalence of avian malaria in the intervening 14 years. Despite considerable effort, we were unable to detect larval *C. quinquefasciatus* and captured only one adult indicating that the vector of avian malaria has a very limited presence at HFNWR. We did, however, document the establishment of another invasive mosquito, *Aedes japonicus japonicus*, and its occurrence in tree fern cavities and rock pools as larval habitat in the lower forests of HFNWR. We suggest that interspecific competition by *A. j. japonicus* and predation by a suite of native predators may provide biotic resistance to the establishment of permanent *C. quinquefasciatus* populations. While current predictions of climate change in the Hawaiian Islands include a gradual warming and enhanced transmission by mid-century, the current cooling trend recorded at high elevation HFNWR illustrates the importance of monitoring to document fine scale temporal and site specific changes in prevalence. Long term changes in precipitation may have a more profound effect on local transmission of malaria than temperature and we may have already seen some potential impacts of an extended drought at HFNWR with a decrease in feral pig activity and pig-associated larval mosquito habitat and increases in stream-associated larval mosquito habitat.

INTRODUCTION

Throughout the main Hawaiian Islands, introduced mosquito-borne avian disease continues to have a profound impact on the distributions and abundance of native Hawaiian forest birds (LaPointe et al. 2012). Avian malaria, *Plasmodium relictum*, and avian pox *Avipoxvirus* spp. likely played key roles in the extinction of a number of endemic Hawaiian bird species since the arrival of the mosquito vector, *Culex quinquefasciatus* in 1826 (Warner 1968, van Riper et al. 1986). In the last fifty years, populations of remaining native forest birds have become largely restricted to high elevation forests where mean temperatures are marginal or inadequate for vector and pathogen development and the limited availability of larval mosquito habitat constrain disease transmission (Scott et al. 1986, Ahumanda et al. 2004, LaPointe et al. 2010). In response to this range contraction, a number of private, state, and federal refuges have been established in these high elevation forests to preserve and restore populations of the remaining Hawaiian forest bird species (Price et al. 2009). Unfortunately, feral pigs (*Sus scrofa*) are also common in these forests and have caused large-scale changes in native vegetation (Cole and Litton 2014). Feral pigs degrade forest bird habitat and contribute significantly to local disease transmission by the creation of larval mosquito habitat (Baker 1975, LaPointe et al. 2009). Therefore, feral pig control and eradication has become a top priority for most refuges in the Hawaiian Islands (Anderson and Stone 1993, Hess et al. 2006). In 1985, Hakalau Forest National Wildlife Refuge (HFNWR) was established for the preservation of endemic avifauna and by 2004 feral pigs had been effectively eliminated from the upper, fence-enclosed portion of the refuge (Hess et al. 2006). Native bird communities at HFNWR remain intact and most species populations are stable or increasing (Camp et al. 2010, Camp et al. 2015, Guillaumet et al. 2015). Early surveys for avian malaria in birds inhabiting HFNWR found minimal evidence of local transmission (Feldman et al. 1995). However, if current local warming trends continue, birds in HFNWR may be exposed to increases in avian disease transmission (Benning et al. 2002, Giambelluca et al. 2008, Fortini et al. 2015, Liao et al. 2015). Recent studies on Kaua`i and at HFNWR have reported increases in avian malaria prevalence among endemic Hawaiian forest birds (Freed et al. 2005, Atkinson et al. 2014, Freed and Cann 2013b). The two- to ten-fold increases in avian malaria prevalence documented on Kaua`i coincided with the rapid population decline in two island endemics, `akikiki (*Oreomystis bairdi*) and `akeke`e (*Loxops caeruleirostris*), and appeared to be associated with climate change across the Alakai Plateau (Foster et al. 2004, USFWS 2010a, Atkinson et al. 2014). Earlier modeling efforts had predicted increases in malaria prevalence on the island of Kaua`i as warming trends relaxed thermal constraints to mosquito-borne transmission (Benning et al. 2002, LaPointe et al. 2010). A 1°C increase in mean daily ambient temperature and shifting patterns in annual precipitation on Kaua`i may have led to higher mosquito densities and increased malarial transmission (Atkinson et al. 2014). At HFNWR (Pua `Ākala, 1,900 m asl) malaria prevalence among resident birds more than doubled from 2.1% in 1994 to 5.4% in 2002 (Freed and Cann 2013b) but the potential impact of increased disease on bird populations remains unclear due to confounding limiting factors and conflicting estimates of population trends (Camp et al. 2010, Freed and Cann 2010). In the last decade, Freed and coworkers (2005, 2008, 2012, 2013 a,b, 2014) have reported declines in Hawai`i `ākepa (*Loxops coccineus*) and Hawai`i creeper (*Oreomystis mana*) populations linked to avian malaria epizootics, explosive population growth of ectoparasites and interspecific competition with Japanese white-eyes (*Zosterops japonicus*). The magnitude of these declines and their impact on long-term population trends has been questioned (Camp et al. 2010, Camp et al. 2015). In addition to the effects of climate change, available funding and staffing in the last decade has limited feral pig control at the refuge (Hess

et al. 2013), potentially resulting in an increase in larval mosquito habitat. A recent Structured Decision Making (SDM) analysis of the long-term, *in situ* preservation of HFNWR avifauna emphasized the need for a current assessment of avian disease transmission at the refuge (Paxton et al. 2012). Managers at HFNWR have identified climate change threats to refuge birds as a key focus of management action, and understanding where and to what extent disease is present on the refuge will be a critical first step for long-term proactive management strategies (USFWS 2010b). The main objective of this study was to compare current avian malaria prevalence and vector mosquito occurrence with data collected 14 years ago (1998–1999) across the broad landscape of HFNWR to determine if avian malaria transmission has changed in the intervening years. We (1) resampled forest birds for malarial prevalence, (2) sampled for adult mosquitoes and (3) surveyed and resurveyed transects for larval mosquito habitat at one high elevation site and two low elevation sites. This study will update the prevalence and distribution of avian disease and vector mosquitoes and help assess future avian disease risk at HFNWR.

METHODS

Study Area

We collected blood samples from forest passerines, trapped adult mosquitoes, and surveyed for feral pig activity and larval mosquito habitat at three sites located within the Hakalau Forest National Wildlife Refuge (Figure 1, Supplemental Maps Appendix I). The refuge is located on the windward (eastern) flank of Mauna Kea Volcano on the island of Hawai`i and encompasses 13,257 hectares of montane rain forest and mesic koa/ōhi`a woodland. Two sites were located at 1,300 m asl; one at the northern end, Maulua (UTMS X: 262098 Y: 2199777; 1,343 m asl) and the other at the southern end, Pua `Ākala (UTMS X: 262476 Y: 2187942; 1,313 m asl) of the refuge. A third site, Nāuhi (UTMS X: 259366 Y: 2197171) was located at 1,598 m asl within the Lower Honohina Tract and the site of an extensive, forest bird demography study from 1994–1999 (Woodworth et al. 2001). The Nāuhi site had been a cattle camp and, later in the 1920's, a state forestry nursery (Swezey and Williams 1932). We also had an upper elevation site at Pua `Ākala (UTMS X: 257204 Y: 2190083) at approximately 1,824 m asl where we conducted terrestrial and stream surveys for larval mosquito habitat and feral pig activity but did not collect blood samples. The lower sites had not been logged or used for cattle ranching, although all the study sites have been variably impacted by feral ungulates and invasive plant species. All sites had been previously sampled between 1998 and 1999 by USGS biologists (DAL and CTA). The upper elevation Nāuhi site lies in a transition zone between wet and mesic forest where mature koa (*Acacia koa*) and ōhi`a (*Metrosideros polymorpha*) are the co-dominant canopy species and hapu`u tree ferns (*Cibotium glaucum*), pūkiawe (*Leptecophylla tameiameiae*), `ōhelo (*Vaccinium reticulatum*) and `ākala (*Rubus hawaiensis*) dominate the understory. First order streams at this elevation are intermittent, flowing after summit snowmelt and island-wide storms. Typically, the stream beds have deeply cut banks with rocky channels. The lower elevation sites lie within wet, montane rain forest where ōhi`a is the dominant canopy species and hapu`u and kōlea (*Myrsine sandwicensis*) dominate the understory. The landscape at the lower elevations is a mosaic of mature forest, open water *Carex* sp./*Juncus* sp. bogs and patches of dieback forest with sparse young ōhi`a and dense mats of uluhe fern (*Dicranopteris linearis*). Streams at this elevation are typically first or second order and perennial under normal precipitation. The rocky banks are shallower and the channel often filled with variable sized cobble. Small waterfalls < 5 m are common along the stream

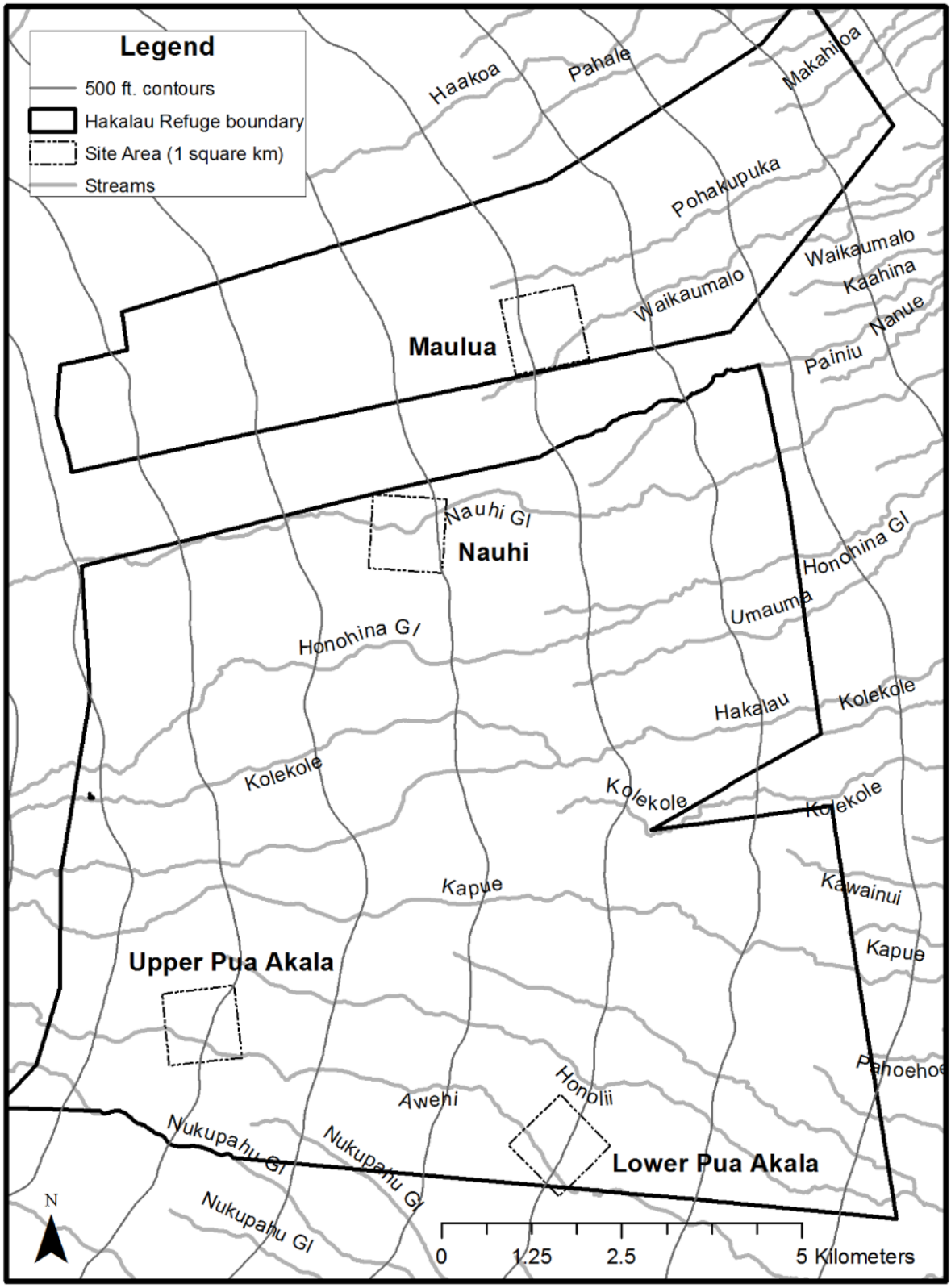

Figure 1. Hakalau Forest National Wildlife Refuge and the four study sites surveyed in 1998 and 2012.

reaches. At the lower Pua `Ākala site, *Sphagnum* sp. moss forms a thick matt under full canopy forests. Though variable from site to site, the forest bird community encountered during this study included: Hawai`i`elepaio, *Chasiempis sandwichensis* (HAEL); `ōma`o, *Myadestes obscurus* (OMAO); Hawai`i `amakihi, *Chlorodrepanis* (*Hemignathus*) *virens* (HAAM); `akiapōlā`au, *Hemignathus wilsoni (munroi* )(AKIA); Hawai`i creeper (HACR); Hawai`i `ākepa (AKEP); `i`iwi, *Drepanis* (*Vestiaria*) *coccinea* (IIWI); `apapane, *Himatione sanguinea* (APAP); Japanese white-eye (JAWE); red-billed leiothrix, *Leiothrix lutea* (RBLE); Japanese bush warbler, *Cettia diphone* (JABW); Northern cardinal, *Cardinalis cardinalis* (NOCA); melodious laughing-thrush, *Garrulax canorus* (MELT); yellow-fronted canary, *Serinus mozambicus* (YFCA); and house finch, *Carpodacus mexicanus* (HOFI).

Mist Netting and Sampling of Avian Blood

Sampling at the Nāuhi site occurred from January 1998 to June 1998 and from January 1999 to May 1999 as part of an extensive forest bird demography study at Hakalau Forest NWR. Mist netting and banding details may be found in Woodworth et al. (2001). Blood samples were collected for diagnostic analysis from 1998–1999 to coincide with work at our other study sites. Sampling of the lower Pua `Ākala and Maulua sites occurred during five, 7–10 day trips made in March 1998, April 1998, May 1998 and February 1999. Sampling at the same geographic locations was repeated from March to August 2012 at Nāuhi and during three, 10 day trips at lower Pua `Ākala (May 2012 and September 2012) and Maulua (October 2012). Only sampling during September 2012 and October 2012 fell within the presumed transmission season of September through December (LaPointe 2000, Samuel et al. 2011). Native and non-native forest birds were captured in mist nets (passively in 1998–1999 and using both passive mist netting and playback recordings in 2012). Both 6 and 12-meter long, 36 mm mesh mist nets supported by double-height (6 m) sections of 2 cm diameter electrical conduit were used during both time periods. In 1998–1999, 12 m aerial nets were hung at a height of 10–15 m at the Nāuhi site. Only one aerial net was set up at Nāuhi in 2012. Up to 15 nets were opened between 0700–0900 h, and checked for birds every 30 min until they were closed at 1600 h. Nets were closed during inclement weather (rain, fog or when the ambient temperature fell below 5°C) or when processing time exceeded one hour. All net locations were mapped using GPS (Garmin GPSMAP® 62S) (Appendix I). Field collections were authorized under the following permits: U.S. Fish & Wildlife Service Threatened and Endangered Species Permit (# TE003483-28) with attached Special Use Permit Guidelines, Hawai`i State Protected Wildlife Permit (# WL-13-07-1), Federal Bird Banding Permit (#22613), and a University of Hawai`i and Institutional Animal Care and Use Committee Protocol (IACUC) (# 09-893-3).

All newly captured birds were weighed and banded with a standard U.S. Geological Survey aluminum band. The lengths of the tarsus, wing, tail, and exposed culmen were measured and standard observations on body condition (furcula fat and feather molt) and breeding status (cloacal protuberance and brood patch) were recorded. Birds were sexed and aged using standard techniques and keys developed for Hawaiian passerines and modified by U.S. Geological Survey biologists (Pyle 2008). While in the hand, birds were examined for lesions characteristic of avian pox and, during 2012, knemidokoptic mange. Presumptive diagnosis of pox infections was based upon the presence of swollen and/or crusty lesions on the feet (early stage) or missing and/or malformed digits (late stage). However, these lesions could also be due to injury, infection by other pathogens or a combination of these factors. Unlike avian malaria, we do not have a simple diagnostic test for *Avipoxvirus*. Still, van Riper et al. (2002) found the descriptive criteria for pox lesions to be 90% accurate when compared with histological examination and/or cultivation of virus from lesions in chicken egg, chorioallantoic

membrane (CAM). Field diagnosis of knemidokoptic mange lesions was also based on descriptive criteria. Knemidokoptic mange lesions were characterized by tassel and scaly growths on the feet of infested birds. Presumptive mange lesions were scraped with a sterile scalpel blade and the scrapings were preserved in 70–95% ethanol and later microscopically examined to confirm the presence of mites or characteristic mite burrows. Whenever suspect pox or mange lesions were encountered, the mist net, banding tools and the bander's hands were immediately disinfected. All mist nets, bird bags, and field equipment (tarps, tents, etc.) were washed and disinfected prior to use in a new study area. During 2012, we examined birds for ectoparasites (chewing lice, skin mites and feather mites) on the feet and five feathered regions: head, breast, rump, primaries and rectrices. Knemidokoptic mange, caused by the skin mite *Knemidokoptes jamaicensis*, was scored as early, intermediate or advanced using standard criteria (Gaudioso et al. 2008). Feather mite densities were scored subjectively for both body (head, breast and rump) and flight (primaries and rectrices) feathers on a scale ranging from 0–3, where 1 = 1–99 individuals, 2 = 100–999 individuals and 3 = 1000+. Specimens of ectoparasites were preserved in 70–95% ethanol for subsequent identification. A blood sample (< 1% of the bird's body weight) was collected from the brachial or jugular veins of all species. Blood smears were made immediately after blood collection, air dried, and fixed in methanol. Remaining whole blood was transferred into microhematocrit tubes and spun in a portable field centrifuge for separation of plasma for serological diagnostics and packed red blood cells for PCR diagnostics. Packed red blood cells were transferred in the field to an equal volume of lysis buffer containing 0.1M tris (hydroxymethyl) aminomethane (Tris), pH 8.0, 0.1 M ethylenediaminetetraacetic acid (EDTA), and 2% sodium dodecyl sulfate (SDS) then held on wet ice until returned to the laboratory and stored at -70°C until diagnostic screening. Blood and plasma samples not destroyed during diagnostics were deposited for long-term storage at the Pacific Island Ecosystems Research Center, Kīlauea Field Station, Avian Disease Laboratory in Hawai`i Volcanoes National Park (HAVO).

Malarial Diagnostics

Microscopy

Blood smears were stained with phosphate buffered (pH 7.0) 6% 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 estimate that we examined approximately 20,000–30,000 erythrocytes. Smears were scored as positive for malaria if we observed at least one infected erythrocyte.

Serology

Plasma samples were tested by immunoblotting to detect antibodies to *P. relictum* using procedures described in detail by Atkinson et al. (2001a). We used plasma from an experimentally infected canary as a positive control in the procedure and included a secondary antibody control that omitted the test plasma to validate method specificity.

Polymerase Chain Reaction (PCR) Analysis

Purified DNA for PCR analysis was extracted from packed blood cells using DNeasy tissue extraction kits (Qiagen Inc., Valencia California) according to 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 EDTA 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 $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate (dNTP), 0.4 µM each primer, and 0.5 units of Promega GoTaq polymerase (Promega North America, Madison, Wisconsin). PCR reactions with primers 343F/496R were run in 25 µl volumes containing the following components in the reaction mix: 2.5 mM $MgCl_2$, 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 and 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 190 bp band. All PCR reactions were run with a positive control consisting of DNA extracted from a Pekin duckling *Anas platyrhynchos domesticus* with an intense experimental infection with *P. relictum* and a negative control that substituted water for DNA. Samples were considered positive for *P. relictum* when an expected 190 bp PCR product was amplified during the second nested reaction.

Infection Status

We combined results from microscopy, serology, and PCR to determine an overall infection status for each bird and to help distinguish acute (microscopy and PCR positive, antibody negative) from chronic (microscopy or PCR negative, antibody positive) infections in native species. Since native birds have lifelong chronic infections detectable by a serological response and we assume PCR can detect parasites even before parasitemia is detectable on a blood smear, we include a third infection class of presumptive acute infection for PCR positive, antibody negative birds where no parasites were detected by microscopy. Low intensity infections are often undetected by microscopy and very low parasitemias, characteristic of non-native species, often go undetected by serology (Atkinson unpublished data). By contrast, some low intensity infections in native species that are not detected by PCR can be detected by serology (Jarvi et al. 2002). If birds tested positive by any of the three methods, we classified the bird as positive for malaria.

Adult Mosquito Trapping

Gravid traps (Model 1712, John W. Hock Company Gainesville, FL) were used for trapping specimens of *C. quinquefasciatus*. An alfalfa infusion was prepared 5 days prior to setting traps. The infusion was made by adding 750 gm of alfalfa rabbit chow and 57 gm of a yeast protein/lactalbumin mixture to 23 L water. All traps were placed within 1 km of the net lanes (Appendix 1). Traps were placed in the shade, close to a tree base or rock to minimize disturbance by feral ungulates. Traps were operated for 4–6 consecutive nights from approximately 1700–0800 h and traps were checked every morning. Mosquitoes were collected using a mouth aspirator and transferred into a 500 ml, waxed paper container fitted with a mesh covering. When possible, collected mosquitoes were maintained on a 3% sucrose solution and taken back to the laboratory for identification and dissection for malarial prevalence. In 1998–1999, we operated traps at our Upper Pua `Ākala, Lower Pua `Ākala and Lower Maulua

site three times throughout the year. We did not sample for adult mosquitoes at Nāuhi in 1998–1999.

Pig Activity, Larval Mosquito Habitat and Stream Surveys

We surveyed four, 1 km long belt transects at each study site to determine the 1) relative frequency of feral pig activity and 2) relative abundance/frequency of available larval mosquito habitat and compared our 2012 results with results from surveys conducted in 1999. Transects ran parallel to slope and were spaced 250–500 m apart (Appendix I). We recorded the presence and age of various pig sign using standard techniques and criteria developed by Anderson and Stone (1993) that have been used to monitor pig activity in HFNWR and other natural areas in the Hawaiian Islands for the past two decades. Although pig activity indices are not a true measure of pig abundance they may be useful in recognizing trends in relative abundance (Nogueira et al. 2007). Procedural details are provided in Appendix II (Standard Operating Procedures) but briefly, 2–3 observers walked downslope on a fixed heading recording the presence and relative age of various pig sign encountered in 100 contiguous plots measuring 10 m x 5 m for each transect. Feral pig sign was recorded by experienced observers trained and calibrated to the primary observer (DAL) to minimize observer error. As we did not sample the Nāuhi site in 1999, we relied on data collected by refuge staff along previously established transects (HFNWR Unit 3 Transects 10B and 11B) for this time period (Hess et al. 2006). All discreet aquatic habitats (any non-flowing, body of water) were also recorded for each transect section. Typical aquatic habitat included, ground pools, pig wallows, tree fern cavities, rock holes and pools in intermittent stream beds (hereafter rock pools), tree holes and occasionally man-made, discarded containers or impoundments. We considered wallows distinct from other ground pools because they are created and frequented by feral pigs. Aquatic habitats were sampled, depending on size, by either extracting the entire water volume (< 3 liters) with a turkey baster or by taking a 250 ml dip sample with a long-handled dipper for every 30 cm^2 of surface area. Water samples were passed through a 250 µm sieve and then the sieve contents were flushed into a sorting tray and examined for mosquito larvae and other macroinvertebrates. Mosquito larvae were identified to species and enumerated. Other insects were identified to family while other invertebrates were identified to higher order taxa and enumerated. Vouchers of specimens were preserved in 70% ethanol for later identification.

We also conducted larval mosquito habitat surveys along a 1,000 m long reach of the closest, accessible stream in each study site (Figure 1, Appendix I). The actual length of the reach surveyed varied due to stream accessibility and observer safety. Stream surveys were conducted in September and October (except Honoli`i Stream in 2012) the period of lowest discharge for windward Hawai`i Island and not during periods of high water when stream banks were inundated. Procedural details are given in Appendix II but briefly, 2–3 observers walked down along the bank or stream channel and recorded the number of discreet rock pools in each 10 m reach of streambed. Sections of the stream channel with running water were excluded. Ten dips with a standard 250 ml, long-handled dipper were sampled from each stream section while attempting to sample all rock pools in the section. Extra dips were taken in wet sections following a dry section of streambed. Mosquito larvae and macroinvertebrates encountered were identified as stated above. Relative abundance was determined by the proportion of dip samples occupied by that species.

Climate Data

Long-term temperature records (> 50 years) from higher elevations are limited on the windward (eastern) Hawai`i Island. We used temperature records from two nearby weather

stations located on the windward slope of Mauna Loa and Mauna Kea volcanoes at elevations above and below our Nāuhi field site at 1,586 m asl and examined the general trends in annual mean, maximum and minimum temperatures. The nearest weather station was located approximately 7 km from the Nāuhi field site in Upper Pua `Ākala at 1,950 m asl and provided a near complete (99%) record of daily temperatures from 2003 to 2013. We used temperature records from the Hawai`i Volcanoes National Park Headquarters station (UTM X: 263103.4, Y: 2149926.1) at 1,200 m asl to examine trends over the same 10-year and a longer 53 year period. We also used long-term precipitation time series from the Hawai`i Volcanoes National Park Headquarters station and Hilo International Airport at 12 m asl. Precipitation data used to evaluate streamflow during stream surveys came from two weather stations (Kahuku UTM X: 274679.6, Y: 2194720.4 and Honomu UTM X: 274844.7, Y: 2196250.9) located downslope from HFNWR at approximately 400 m asl. Temperature and precipitation data was accessed from the Western Region Climate Center (http://www.raws.dri.edu/wraws/hiF.html) and the NOAA National Climatic Data Center (http://www.ncdc.noaa.gov/).

Statistical Analysis

We used a Pearson Chi-square test (Systat Statistical Software Version 13.1, 2015) to assess the change in the overall prevalence of malaria infection in forest birds at HFNWR between 1999 and 2012 and to assess changes in the proportion of individual species represented in each period sample. We also used Fisher exact tests and Chi–square tests to test for age associations with infection status. Due to small sample sizes, we combined data from the two low elevation sites. We constructed a series of models and used logistical regression to test for association between the independent covariates (*year*, *site*, *host species*) and a single interaction *year*site*) on the probability of malarial infection and used corrected Akaike's information criterion (AICc) for model selection (Program R, R Core Team, Version 3.1.3,2015). We included the *year*site* interaction in the initial model because a significant interaction between *site* and *year* would be expected if effects from climate change (or management actions) were variable between altitudinal sites over the intervening years. For example, if a general warming trend had increased malarial transmission at the lower elevation sites but was still inadequate to increase transmission at the cooler high elevation site.

We used a Pearson Chi-square test (Systat Statistical Software Version 13.1, 2015) to assess the change in feral pig activity at each site between 1999 and 2013. Change was based on the proportion of 200–400 plots where recent feral pig sign was detected. As we did not survey the Nāuhi site in 1999, we used 1999 data collected by refuge staff on two of our four kilometer long transects. Since our main observer (DAL) was trained by refuge staff, the protocols were similar for surveys conducted in 1999 and 2012. We used a paired T-test to compare the abundance of rock holes in stream beds between 1999 and 2012 assuming that the stream bed topography had not changed significantly in 13 years. We used Fisher exact tests to compare the presence of tree fern cavities, wallows and ground pools between sampling periods. Trends in the temperature data were examined by line smoothing (5-year moving average) and linear regression (H_0: slope = 0). Confidence intervals for proportions were calculated using the Wilson score method (Brown et al. 2001). All tests were considered significant at alpha ≤ 0.05 with Bonferroni corrections applied for multiple comparisons where necessary.

RESULTS

Mist Netting and Disease Prevalence

Between January 24, 1998 and May 14, 1999, over 800 passerines birds representing 10 species were captured or recaptured in 5,966 net hours at Nāuhi. We collected 422 blood samples for malarial diagnostics. During this first sampling period, we also mist-netted forest birds at lower Pua `Ākala from March 10–17, 1998 and February 18–23, 1999. We completed 1,425 net hours at lower Pua `Ākala and captured 104 passerines birds; 60 (58%) of which were native species. `I`iwi and non-native Japanese white-eyes made up 51% of all birds captured at lower Pua `Ākala. We also mist-netted for 1,498 net hours at lower Maulua from April 8–9 and April 27–May 3, 1998 and February 11–16, 1999. We caught and bled a total of 102 passerine birds; 71 (70%) of which were native species. Hawai`i `amakihi and Japanese white-eyes made up 44% of the sample. We did not detect Hawai`i creeper, Hawai`i `ākepa or `akiapōlā`au during the 12–13 days we camped at each of the lower elevation sites in 1998 to 1999, despite the participation of well-trained observers. During our second sampling period we completed 2,733 net hours of mist-netting at Nāuhi between March 27 and August 8, 2012. At Nāuhi we captured 304 passerines birds representing 11 species. Eighty-six percent were native species with `i`iwi and Hawai`i `amakihi accounting for more the half (59%) of the Nāuhi sample (Table 1). We also collected 284 individual blood samples, each yielding a separate blood smear, plasma, and whole cell sample. From May 24–26, 2012 and again from August 28–September 4, 2012 we completed 802 net hours of mist netting at the lower Pua `Ākala site. We captured a total of 35 birds, 23 (66%) of which were native species, and collected 34 individual blood samples. The most frequently captured species at lower Pua `Ākala was the Hawai`i`elepaio. We also captured a hatch year, endangered Hawai`i creeper at lower Pua `Ākala and made aural detections of Hawai`i creepers and Hawai`i `ākepa and a visual detection of an `akiapōlā`au on the study site. From October 10–17, 2012 we completed 666 net hours of mist netting at the lower Maulua site and captured 53 birds. Fifty-one percent of total captures were native species (N = 27). The most frequently captured species were the red-billed leiothrix and `i`iwi which accounted for more than half (55%) of all birds captured at Maulua. Fifty-three blood samples were collected from birds at lower Maulua. No endangered species were captured but aural detections of Hawai`i creeper and visual and aural detections of `akiapōlā`au were made on or within 1 km of the study site.

Composition of the forest bird community sampled from the all sites was similar between the sample periods for most native species (Table 1). Proportionately fewer Hawai`i `amakihi were represented in the lower elevation sites in 2012 but the overall proportion of resident native, native and non-native birds did not change. At Nāuhi proportionally fewer Hawai`i `elepaio and red-billed leiothrix were sampled in 2012. There was little change among resident native, native and non-native birds. We captured a single yellow-fronted canary and Japanese bush warbler at Nāuhi in 2012. Neither species was previously captured at Nāuhi in 1998–1999. At the lower Pua `Ākala and Maulua sites, sample sizes for many species were too small for statistically valid year to year comparisons. Proportionately fewer Japanese white-eyes and `i`iwi were represented in the 2012 sample from lower Pua `Ākala and proportionally fewer Hawai`i `amakihi were in the 2012 sample from Maulua. We combined the low site data for further analysis and found lower proportions of Hawai`i `amakihi and Japanese white-eyes in the 2012 sample. Among native species sampled the proportion of hatch year (HY) birds was 6% (17/291) in 1998 and 8% (43/512) in 2012 (Appendix III). There was no difference in the ratio between juvenile and adult birds ($X^2 = 1.75$, df = 1, P = 0.185). When after hatch year (AHY)

birds are included with HY birds the proportion of juvenile (HY and AHY) birds was 30% (152/512) in 1998 and 18% (51/291) in 2012.

Table 1. Species composition in the sampled avian community from 1998 to 2012 at Hakalau Forest NWR.

Species	Percentage of total avian community sampled[a]					
	Nāuhi		low sites		all sites	
	1998 N = 366	2012 N = 276	1998 N = 194	2012 N = 84	1998 N = 560	2012 N = 360
`i`iwi	29.2	32.2	16	15.5	24.6	28.3
`apapane	12.3	10.9	14.4	6*	13	9.7
Hawai`i `amakihi	21	28.6	16	11.9**	19.3	24.7*
Hawai`i `elepaio	12.3	6.9*	8.8	20.2	11.1	10
`ōma`o	6.6	8.7	8.3	4.8	7.1	7.8
Japanese white-eye	4.9	7.6	24.2	13.1*	11.6	8.9
red-billed leiothrix	13.7	5.1***	12.4	28.6*	13.2	10.6
resident natives	39.9	44.2	33	36.9	37.5	42.5
all natives	81.4	87.3*	63.4	58.3	75.2	80.6
non-natives	18.6	12.7*	36.6	41.7	24.8	19.4

[a]Species with fewer than 20 total individuals sampled were removed from the analysis including endangered native birds and non-natives. *P ≤ 0.05, ** P ≤ 0.01, ***P ≤ 0.001

`Apapane (19.4%), `ōma`o (17.6%) and Japanese white-eyes (14.3%) had the highest overall prevalence of infection. We also detected infected individuals among Hawai`i `amakihi (9.6%), `i`iwi (6.3%) and Hawai`i `elepaio (6.1%) as well as a single house finch from Nāuhi (Figure 2, Table 2). Malaria infection was not detected in endangered Hawai`i `ākēpa (N = 7), `akiapōlā`au (N = 4), Hawai`i creeper (N = 17) or non-native northern cardinal (N = 3), melodious laughingthrush (N = 1), Japanese bush warbler (N = 1) or yellow-fronted canary (N = 1). Most infections (97.7%) were detected by PCR and more than half (58.6%) of these were confirmed by serology and/or microscopy (Table 3) to distinguish between acute and chronic infections. Among resident native birds (Hawai`i `amakihi, Hawai`i `elepaio, `ōma`o), there was no significant association between malarial prevalence and age class (after hatch year (AHY) birds vs. second year (SY) and older birds) at Nāuhi in 1998 ($X^2 = 0.02$, P = 0.89), combined low sites in 1998 ($X^2 = 0.11$, P = 0.74), Nahui in 2012 ($X^2 = 0.13$, P = 0.72), or combined low sites in 2012 ($X^2 = 0.88$, P = 0.35). During the 1998–1999 sampling period, 19 birds (Nāuhi N = 8, lower Pua `Ākala N = 5, Maulua N = 6) were recaptured and re-bled across a 9–14-month interval that would have included the transmission season. Only one bird changed infection status. A Hawai`i `amakihi captured at Nāuhi that tested positive by PCR and immunoblot in 1998 was found negative upon re-testing a year later in 1999. Similarly, we found no change in the infection status in seven additional birds recaptured between one and five months later. We also detected no change in the infection status of 51 birds recaptured in 2012. Most recaptures (N = 41) were made between one week and three months later but 10 birds were recaptured at greater than three months.

Chronic infections (antibody positive) made up 73.2% (52/71) of all positive birds. Acute infections with high parasitemias were rare. An `i`iwi captured in April at Nāuhi had an acute parasitemia but tested negative by immunoblot. We detected presumptive acute infections (positive for the PCR reaction but negative for immunoblot test and microscopy) in 14 native birds. These birds represent new infections before parasitemias are detectable and prior to an immune response. Hawai`i `amakihi with presumptive acute infections were captured at Maulua in 1998 (N =1) and 2012 (N = 1) and at Nāuhi in 1998 (N = 3) and 2012 (N = 1). `Apapane with presumptive acute infections were captured at Nāuhi in 1999 (N = 1) and in 2012 (N = 1). We also detected presumptive acute infections in `i`iwi at Nāuhi in 1998 (N = 3) and Maulua in 1999 (N = 2). In 2012, an `ōma`o with a presumptive acute infection was captured at Nāuhi.

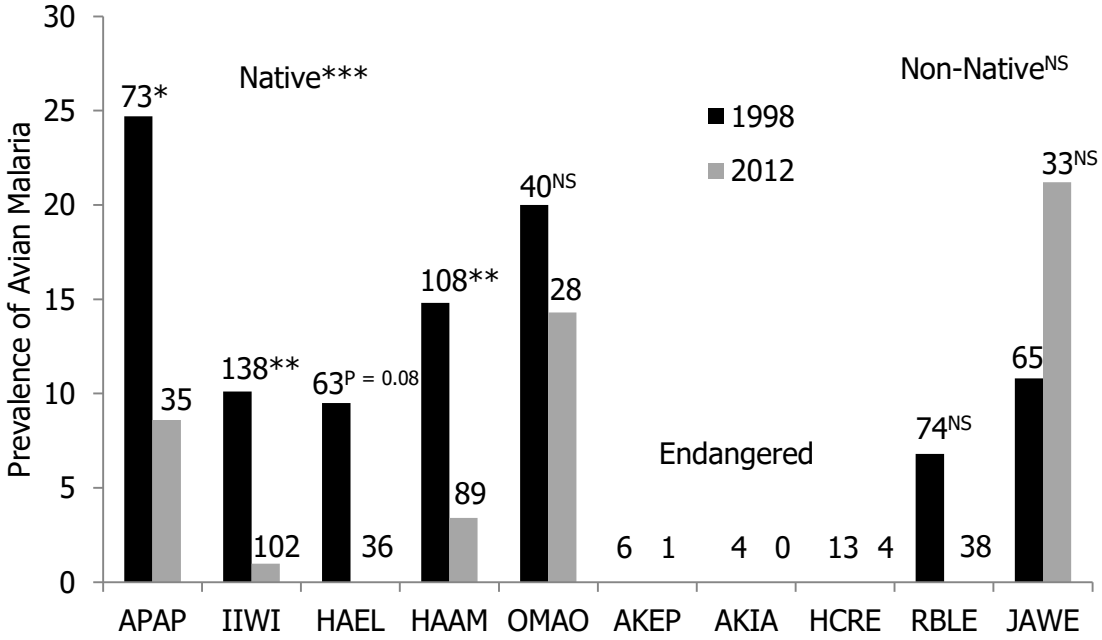

Figure 2. Change in the prevalence of avian malaria in forest birds at Hakalau Forest NWR sampled between 1998-1999 and 2012. Sample sizes are shown above bars. Pearson Chi-square or Fisher's exact test * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001, NS = Not Significant

The overall prevalence of avian malaria differed significantly across our three sites in 1998 (X^2 = 7.01, P = 0.03) but not in 2012 (X^2 = 4.16, P = 0.13) (Figure 3). In 1998 prevalence differed between Nāuhi and Maulua (X^2 = 6.87, P = 0.01) but not between Nāuhi and Pua `Ākala (X^2 = 1.26, P = 0.26) or between the two lower sites (X^2 = 1.17, P = 0.28). The overall prevalence of avian malaria in all forest birds from the three sites combined decreased significantly from 12.4% (72/583) in 1998 to 4.9% (18/371) in 2012 (X^2 = 14.92, df =1, P < 0.001). Prevalence in the entire avian community decreased by a factor of two or more at all three sites although comparisons at low elevation sites (Pua `Ākala and Maulua) were based on small sample sizes in 2012 (Mantel-Haenszel X^2 = 12.86, P < 0.001) (Figure 3).

In general, the prevalence of malarial infection decreased in native species over the intervening 14 years (Figure 2, Table 2, Appendix IV). Significant decreases between sampling years were seen among all native species (X^2 = 20.85, P = 0.000), resident native species (X^2 = 9.1, P = 0.003), vagile species (X^2 = 13.4, P = 0.000), Hawai`i `amakihi (X^2 = 7.33, P = 0.007), `apapane ($X^2$ = 3.91, P = 0.048), and `i`iwi ($X^2$ = 8.41, P = 0.004). Malaria prevalence from 1998 to 2012 did not change significantly for `ōma`o (FET, P = 0.75), Hawai`i `elepaio (FET, P = 0.082), red-billed leiothrix (FET, P = 0.17), or Japanese white-eye (FET, P = 0.22). Prevalence among all non-native birds remained unchanged (X^2 = 0.008, P = 0.929).

Table 2. Avian malaria prevalence (%) by species and sampling period.

Common Name	1998		2012		Overall	
	%	(NP/TN)[a]	%	(NP/TN)	%	(NP/TN)
Native Species						
`i`iwi	10.1	(14/138)	0.99	(1/102)	6.3	(15/240)
Hawai`i `amakihi	14.8	(16/108)	3.4	(3/89)	9.6	(19/197)
`apapane	24.7	(18/73)	8.6	(3/35)	19.4	(21/108)
Hawai`i elepaio	9.7	(6/62)	0	(0/36)	6.1	(6/99)
`ōma`o	20	(8/40)	14.3	(4/28)	17.7	(12/68)
`ākepa	0	(0/6)	0	(0/1)	0	(0/7)
`akiapōlā`au	0	(0/4)			0	(0/4)
Hawai`i creeper	0	(0/13)	0	(0/4)	0	(0/17)
Total native	14	(62/444)	3.7	(11/295)	9.6	(71/737)
Non-Native Species						
red-billed leiothrix	6.8	(5/74)	0	(0/38)	4.5	(4/112)
Japanese white-eye	10.6	(7/65)	21.9	(7/32)	14.3	(14/99)
northern cardinal	0	(0/2)	0	(0/2)	0	(0/3)
melodious laughingthrush	0	(0/1)			0	(0/1)
Japanese bush warbler			0	(0/1)	0	(0/2)
house finch	100	(1/1)			100	(1/1)
yellow-fronted canary			0	(0/1)	0	(0/1)
Total non-native	9.1	(13/143)	9.5	(7/74)	9.2	(20/218)
Totals	12.8	(75/587)	4.9	(18/369)	9.7	(93/958)

[a] NP/TN = Number testing positive for malaria/Total number tested.

The logistic regression model we tested (*infection* ~ *year* + *site* + *species* + *site:year*) contained the main categorical covariates *year*, *site* and *species* and the single interaction term, *year *site.* We used 2012 as the reference *year* and Nāuhi as the reference *site.* Red-billed leiothrix was used as the reference *species* as previous studies indicated this species had the lowest malarial prevalence. Species represented by fewer than 20 individuals were dropped from the analysis as were interaction terms containing both *species* and *site* to prevent quasi-complete separation of the data. Malaria was not detected in our limited sample of birds from lower Pua `Ākala in 2012 and this led to further complications with inclusion of interaction terms. However, we did include the *site:year* interaction in the full model because we felt this was a particularly relevant term. For example, an interaction between *year* and *site* might be

Table 3. Summary of all malarial infections by species and diagnostic technique for all sites at Hakalau Forest NWR 1998–1999, and 2012. Columns under diagnostics represent number testing positive, total number tested and the percent positive.

Species	Total #	Microscopy	Serology	PCR
HOFI	1	0 (1) 0%	0 (1) 0%	1 (1) 100%
RBLE	5	0 (5) 0%	0 (4) 0%	5 (5) 100%
JAWE	14	1 (13) 7.7%	1 (12) 8.3%	14 (14) 100%
HAEL	6	0 (5) 0%	5 (5) 100%	6 (6) 100%
OMAO	12	1 (12) 8.3%	11 (12) 91.6%	11 (12) 92%
IIWI	15	1 (13) 7.7%	5 (12) 41.7%	15 (15) 100%
HAAM	19	1 (19) 5.3%	12 (19) 63.2%	18 (19) 100%
APAP	21	8 (18) 44.4%	17 (19) 89.5%	21 (21) 100%
TOTAL	93	12 (86) 14%	51 (84) 60.7%	90 (93) 96.8%

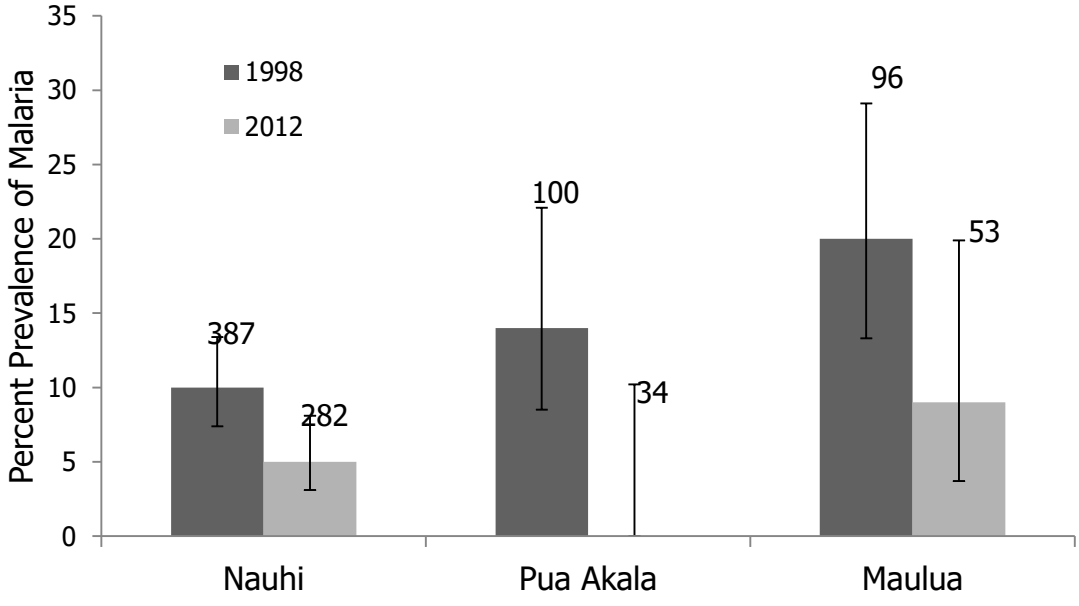

Figure 3. Change in avian malaria percent prevalence (± CI's) at three sites at Hakalau Forest NWR between 1998 and 2012. Sample sizes are shown above bars.

Table 4. Candidate models, AICc values and Akaike weights for evaluating effects of Year, Species and Site on malarial prevalence.

Model	K	Log-Like.	AICc	ΔAIC	Weight
Malaria ~ Year + Species	8	-277.95	572.05	0	0.534
Malaria ~ Site + Year + Species + Site:Year	12	-274.01	572.36	0.31	0.457
Malaria ~ Site + Year	4	-287.01	582.07	10.02	0.004
Malaria ~ Site + Year + Site:Year	6	-285.19	582.46	10.41	0.003
Malaria ~ Year	2	-290.11	584.24	12.19	0.001
Malaria ~ Site + Species + Year	10	-275.84	585.09	13.04	<0.001
Malaria ~ Site + Species	9	-283.45	585.09	13.04	<0.001
Malaria ~ Species	7	-286.45	587.11	15.06	<0.001
Malaria ~ Site	3	-295.36	596.75	24.7	<0.001
Malaria ~ Intercept Only	1	-299.08	600.16	28.11	<0.001

expected if the lower sites differed from our upper site because of changes in climate or feral pig management over the 14-year period. Candidate models, AICc and model weight values are given in Table 4. The best fit model, *year + species*, was slightly better than the full model. The model had a null deviance of 598.15 (df = 919), a residual deviance of 555.89 (df = 912) and an AICc value of 572.05. *Year* and *species* proved to be significant covariates ($P < 0.05$) (Table 5). During our 1998 sampling period, forest birds were 2.87 times more likely to be infected with avian malaria than in 2012 (OR = 2.87, 95% CI = 1.71–5.07, P = 0.0001). Among bird species, `apapane (OR = 5.23, 95% CI = 2.02 – 16.25, P = 0.0015) and `ōma`o (OR = 5.03, 95% CI = 1.75–16.60, P = 0.0041) were significantly more likely than red-billed leiothrix to have avian malaria. Hawai`i `amakihi were 2.57 times more likely than red-billed leiothrix to be infected but the relationship was barely significant (OR = 2.57, 95% CI =0.99–7.96, P = 0.0703).

Along with malaria, we also observed pox-like lesions (early and/or late stage) on native and non-native birds at all study sites (Table 6). Of the 1,004 birds examined, 50 (5%) had early or late stage pox-like lesions. Presumptive pox prevalence was similar among native birds (4.5–7.5%) and slightly less for non-native birds (2–3.4%). The highest prevalence was seen in Hawai`i `amakihi (7.5%) and `ōma`o (7.0%) and the lowest prevalence was seen in the non-native Japanese white-eye (2.0%). Lesions on resident native birds made up 52% (26/50) of all presumptive pox cases observed but the proportion did not differ significantly from pox in vagile species (36% (18/50); $X^2 = 2.6$, df = 1, P = 0.107). Forty percent (20/50) of all pox-lesions were early stage. We observed early or active pox on `i`iwi (N = 2), `apapane (N =1), Hawai`i `amakihi (N = 4) and red-billed leiothrix (N = 4) at Nāuhi in 1998 and on `i`iwi (N= 3), `apapane (N = 1), Hawai`i `amakihi (N = 1), `ōma`o (N = 1), Hawai`i `elepaio (N = 1) and red-billed leiothrix (N = 1) at Nāuhi in 2012. Early stage pox-like lesions were not observed at Pua `Ākala or Maulua in 1998–1999 but were observed on Hawai`i `amakihi (N = 1) and `i`iwi (N = 1) from these sites respectively in 2012. The prevalence of early pox-like lesions did not differ between sampling periods at Nāuhi (1998 2.3% vs. 2012 2.8%; $X^2 = 0.194$, df =1, P = 0.66) or between elevation sites in 2012 (Nāuhi 2.8% vs. Combined low sites 2.2%; $X^2 = 0.081$, df = 1, P = 0.776). Among the 93 birds that tested positive for avian malaria, 5 (5.4%) birds also had pox-like lesions.

Table 5. Model estimates for covariate effects on the probability of malaria infection in forest birds sampled at Hakalau Forest NWR in 1998 and 2012.

Covariates	Estimate	S.E.	95% CI		P value
Intercept	-3.8617	0.514	-4.9932,	-2.9465	0.0000***
Year					
1998	1.0556	0.2756	0.5370,	1.6233	0.0001***
Species					
APAP	1.6544	0.5211	0.7039,	2.7883	0.0015**
HAAM	0.9620	0.5204	-0.0088,	2.0740	0.0703
HAEL	0.3607	0.6246	-0.8751,	1.6384	0.5636
IIWI	0.4331	0.5320	-0.5494,	1.5812	0.4155
JAWE	1.2923	0.5441	0.2817,	2.4592	0.0175*
OMAO	1.6151	0.5620	0.5612,	2.8093	0.0041***

We examined 256 individual birds including 59 Hawai`i `amakihi for ectoparasites in 2012. We found no evidence of knemidokoptic mange (*Knemidokoptes jamaicensis*) on birds examined at HFNWR but feather mites were present on all species. *Analges* sp. feather mites were found on the head or body of 23% (59/256) of all birds. *Proctophyllodes* sp. feather mites, which are restricted to the primaries and rectrices, were only found on 5% (12/256) of all birds examined. The prevalence of ectoparasites in birds was greater at Nāuhi (32%, 56/174) than at either Maulua (12%, 6/50; $X^2 = 7.904$ df = 1, P = 0.0049) or Pua `Ākala (19%, 6/32; $X^2 = 2.319$, df = 1, P = 0.1278). Among individual species with more than 20 observations, `i`iwi (43%, 32/74) and `apapane (53%, 16/50) had a higher prevalence of ectoparasites than Hawai`i `amakihi (7%, 4/59), Hawai`i `elepaio (17%, 4/24), Japanese white-eye (14%, 3/21) or red-billed leiothrix (7%, 2/28). Chewing lice (Phthiraptera: Mallophaga) were not detected on native birds at HFNWR. The chewing louse, *Picicola* sp., was only found on red-billed leiothrix (N = 2) at Nāuhi.

Adult Mosquito Trapping

Between 1998 and 1999, we operated 5–6 gravid traps for 2–6 nights at each site multiple times throughout the year for a total of 161 trap-nights (TN). No adult mosquitoes were captured or encountered during that time. In 2012, we operated 1–6 gravid mosquito traps for 3–6 nights at each site for a total of 146 trap-nights and caught only one *C. quinquefasciatus* (Table 7). That mosquito was captured at the Lower Pua `Ākala during our August/September trip. We had trapped at Lower Pua `Ākala earlier in May but did not capture any mosquitoes at that time. Both Upper Pua `Ākala and Lower Maulua were only trapped once in September and October, respectively, but these months are when *C. quinquefasciatus* populations peak. Traps were operated on four occasions at the Nāuhi site in March and April 2012 and again in August and September 2012. While we did not observe *C. quinquefasciatus* or *Aedes albopictus* adults in the field, host seeking *Aedes japonicus japonicus* adults were frequently encountered at both the Lower Pua `Ākala and Lower Maulua sites. No adult mosquitoes were observed at the high elevation Nāuhi or Upper Pua `Ākala sites.

Feral Pig Activity and Available Larval Mosquito Habitat

We observed a significant decrease in feral pig activity in three of our four study sites over the intervening years (Figure 4). This decrease was particularly evident at the two mid elevation sites at Maulua (X^2 = 263.826, df = 1, P < 0.000) and Lower Pua `Ākala (X^2 = 129.501, df = 1, P < 0.000) where pig sign was observed 30–35 times more in 1999. Feral pig activity also decreased at the Nāuhi site (X^2 = 26.377, df = 1, P < 0.000) though not to the degree seen in our lower elevation sites. Conversely, feral pig activity increased dramatically at the Upper Pua `Ākala site (X^2 = 95.46, df = 1, P < 0.000) where there had been virtually no feral pig activity in 1999.

Feral pig-created tree fern cavities were the most often encountered larval mosquito habitats on our transects comprising 72.8% (209/287) of all available larval mosquito habitat and occurring in 2–10% of all transect quadrants depending on site (Figure 5 A). Wallows, however, were rare throughout the refuge occurring on less than 1% of quadrants and comprising only 3.5% (10/287) of all available larval mosquito habitats (Figure 5 B). Ground pools made up 22.7% (65/287) of total available larval mosquito habitat and, depending on site, occurred on less than 1 to 5% (Figure 5 C) of quadrants. We also found a tree hole in the trunk of toppled koa, a rock pool where the transect crossed a stream bed and a small, plastic cistern. These extremely rare habitats made up only 1% (3/287) of all available larval mosquito habitats.

In general, larval mosquito habitat was more prevalent at lower elevation sites than at higher elevation sites in 1999 and decreased over the intervening years (Figure 5). Feral pig-created, larval mosquito habitat was more common in Maulua than Pua `Ākala where natural wetlands, like temporary ground pools, bogs, and rock pools along intermittent stream beds, were more common. Decreases in available larval habitat over time was most evident for tree fern cavities at Maulua (P = 0.007). Prevalence of pig wallows and ground pools also decreased over the intervening years but these differences were not statistically significant. While the prevalence of most available larval habitat decreased over time the prevalence of tree fern cavities at Upper Pua `Ākala (P < 0.001) increased dramatically in 2012. In 1999, we examined 132 potential larval mosquito habitats (89 tree fern cavities, 28 ground pools, 8 rock pools, 5 wallows, 1 pond and 1 tree hole) from three sites but did not find mosquito larvae (*C. quinquefasciatus* or *A. albopictus*). We examined 76 potential larval mosquito habitats (37 tree fern cavities, 30 ground pools, 5 rock pools, 3 wallows and 1 cistern) in 2012 and found 2 tree fern cavities with *A. j. japonicus* larva.

Table 6. Prevalence of pox-like lesions among forest birds at Hakalau Forest NWR during 1998–1999 and 2012 surveys. Prevalence (Number infected/Total number examined)

Site/Elevation	Year	HAAM	APAP	IIWI	OMAO	HAEL	OTHER[a]	JAWE	RBLE	Total
Nāuhi 1598 m	1998	9.6% (8/83)	2.0% (1/49)	6.2% (7/113)	3.7% (1/27)	3.6% (2/56)	0% (0/30)	5.0% (1/20)	5.5% (3/55)	5.4 % (23/433)
	2012	3.9% (3/77)	6.7% (2/30)	5.6% (5/90)	12.5% (3/24)	5.3% (1/19)	0% (0/7)	4.8% (1/21)	7.1% (1/14)	5.7% (16/282)
Maulua 1343 m	1998	4.2% (1/24)	12.5% (2/16)	0% (0/12)	0% (0/9)	0% (0/6)	NC	0% (0/19)	0% (0/11)	3.1% (3/97)
	2012	25.0% (1/4)	0% (0/5)	8.3% (1/12)	0% (0/1)	20.0% (1/5)	0% (0/1)	0% (0/8)	0% (0/17)	5.7% (3/53)
Pua `Ākala 1313 m	1998	0% (0/7)	0% (0/12)	0% (0/21)	0% (0/7)	15.4% (2/13)	0% (0/1)	0% (0/30)	0% (0/13)	1.9% (2/104)
	2012	33.3% (2/6)	NC	0% (0/1)	33.3% (1/3)	0% (0/13)	0% (0/2)	0% (0/3)	0% (0/7)	8.6% (3/35)
Total		7.5% (15/201)	4.5% (5/112)	5.2% (13/249)	7.0% (5/71)	5.4% (6/112)	0% (0/41)	2.0% (2/101)	3.4% (4/117)	5.0% (50/1004)

[a]OTHER includes rare endangered honeycreepers and uncommon non-native species.

Table 7. Adult mosquito trapping effort in 1998, 1999, and 2012 at Hakalau Forest NWR.

Month	Nāuhi			Upper Pua `Ākala			Lower Pua `Ākala			Lower Maulua		
2012	Traps	Nights	TNs*	Traps	Nights	TNs	Traps	Nights	TNs	Traps	Nights	TNs
Mar	1	3	3	-	-	-	-	-	-	-	-	-
Apr	1	3	3	-	-	-	-	-	-	-	-	-
May	-	-	-	-	-	-	2	6	11	-	-	-
Aug	5	3	15	-	-	-	6	6	36	-	-	-
Sep	6	4	24	6	4	24	-	-	-	-	-	-
Oct	-	-	-	-	-	-	-	-	-	6	5	30
Total			45			24			47			30
(1998) -1999												
Jan	-	-	-	5	2	8	-	-	-	-	-	-
Feb	-	-	-	-	-	-	-	-	-	-	-	-
Mar							(6)	(4)	(18)	(6)	(5)	(30)
Jun	-	-	-	6	4	24	6	6	23	-	-	-
Sep	-	-	-	-	-	-	6	4	24	-	-	-
Oct	-	-	-	6	4	22	-	-	-	6	5	30
Total			-			54			47			60

*TNs = Traps x Nights - Malfunctions.

Stream Surveys

Sampling effort and the prevalence of mosquito larvae and major predatory taxa are summarized in Table 8. In October 1999 we surveyed a 1,000 m reach of `Āwehi Stream and a 450 m reach of Honoli`i Stream starting at approximately 1,800 m asl. We also surveyed a 1,000 m reach on both the `Āwehi and Waikaumalo Streams starting at around 1,300 m asl. In September and October 2012 we resurveyed 800 m of Waikaumalo Stream, 640 m of the lower section of `Āwehi Stream and, in December 2012, 1,000 m of Honoli`i Stream. We also surveyed a 500 m reach of Nāuhi Gulch starting at approximately 1,600 m asl. We found rock pools suitable for larval mosquito habitat, along all reaches of the stream beds surveyed at mean densities ranging from 1 (Nāuhi Gulch) to 8.5 (lower `Āwehi Stream) rock pools per 10 m reach (Figure 6). Not surprisingly, rock pool abundance fluctuated between stream sites and sampling periods. Rock pool abundance decreased along the Honoli`i reach ($P = 0.0015$) and increased along the Waikaumalo reach ($P = 0.0002$) between sampling periods. While we do not have on-site precipitation and streamflow data to compare stream conditions at the time of our sampling, rainfall totals from the nearest NCDC weather station for the 7 and 14 days prior to the 2012 stream sampling suggests drier overall conditions. Rainfall data for seven days prior to the 2012 sampling on the Honoli`i and Waikaumalo Streams were 5 to 40 times less, respectively, than during the 1999 survey (Table 9). Discharge data from the Honoli`i Stream (Table 9) depicts a corresponding period of lower streamflow, except on the one day of sampling on the upper Honoli`i during heavy rain (January 18, 2013).

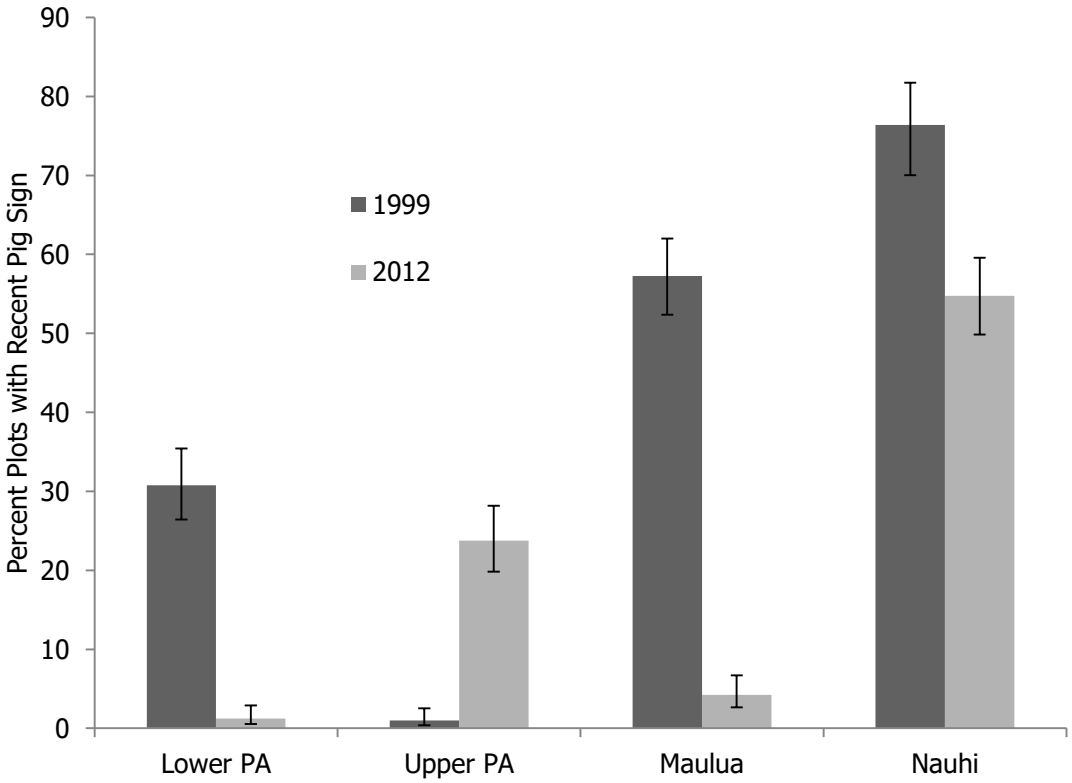

Figure 4. Change in percent feral pig activity (± CI's) at four sites at Hakalau Forest NWR between 1999 and 2012 (95% confidence intervals were calculated by the Wilson score method; Wilson 1927).

Although over 1,000 individual rock pools were sampled during each time period, we found no *C. quinquefasciatus* larvae. However, we did detect larvae of *A. j. japonicus* in the lower reaches of both the `Āwehi and Waikaumalo Streams during our recent 2012 survey. *Aedes japonicus japonicus* larvae were rare, however, occurring in < 1% of dip samples examined. The most dominant predatory taxa included the veliid *Microvelia vagans* present in 34–52% of sample dips and the copepods *Acanthocyclops vernalis* and *Macrocyclops albidus* present in 2.9–55% of sample dips from all streams and elevations surveyed. A number of damselfly and dragonflies larvae were found in rock pools including the endemic *Megalagrion calliphya*, *Megalagrion blackburni*, and *Anax strenuus*. Other predacious invertebrates included the green hydra *Hydra viridissima* which was common in the high elevation drainages of Honoli`i Stream and Nāuhi Gulch and the endemic diving beetle *Rhantus pacificus* which was present in all stream reaches sampled.

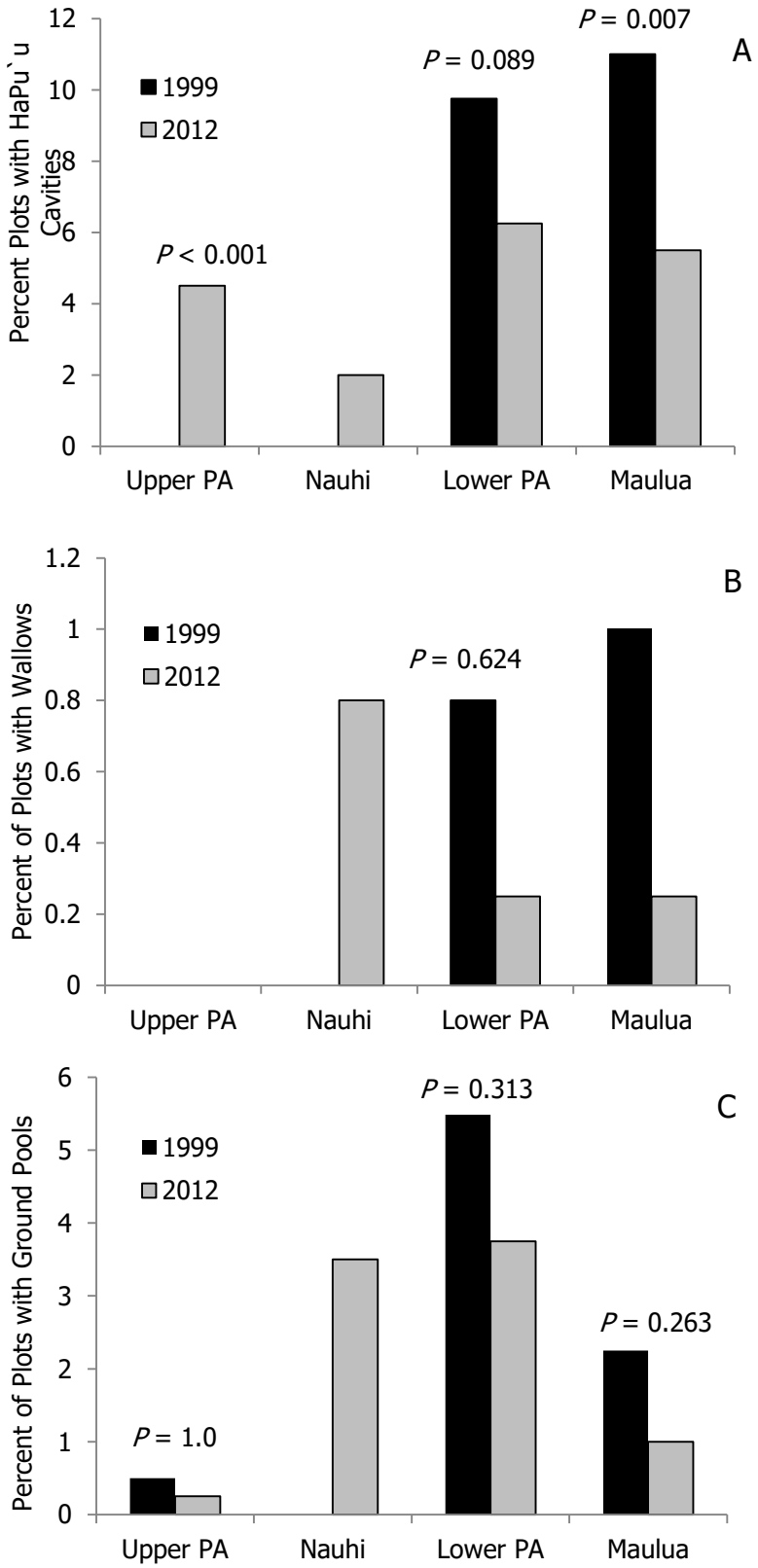

Figure 5. Percent presence of available larval mosquito habitat (N = 400 plots) at Hakalau Forest NWR, 1999 and 2012. A) Tree fern cavities, B) Wallows and C) Ground pools.

Climate Change

At Hawai`i Volcanoes National Park (HAVO), maximum ($F = 19.91$, $P < 0.000$) and mean ($F = 13.17$, $P = 0.001$) temperatures significantly increased over the last 50 years at a rate of 0.134°C per decade (Figure 7). During that time, mean annual temperatures increased by nearly 0.7°C. However, line smoothing (5-year moving average) of the series revealed a cycle and a downward trend over the last decade from 2003 to 2013. Significant negative trends in mean annual temperatures were detected at both HAVO (-1.025°C/decade, $F = 19.238$, $P = 0.002$) and HFNWR (-1.212°C/decade, $F = 51.985$, $P < 0.000$) between 2003 and 2013 (Figure 8). Minimum and maximum temperatures at HAVO and minimum temperatures at HFNWR also had decade-long negative trends while the mean annual maximum temperature at HFNWR remained fairly constant.

Table 8. Summary of stream surveys conducted at Hakalau Forest NWR in 1999 and 2012.

Stream	Year	Reach (m)	# Rock pools	Total Dips	# Dips (%) *Aedes japonicus*	# Dips (%) *Microvelia vagans*	#Dips (%) Copepoda[1]	# Dips (%) Odonata[2]
Nāuhi Gulch	2012	500	51	281	0 (0)	25 (8.9)	27 (9.6)	0 (0)
Honoli`i Stream	2012	1000	280	811	0 (0)	182 (22.4)	325 (40.1)	0 (0)
	1999	450	136	450	0 (0)	229 (50.9)	249 (55.3)	1 (0.2)
Waikaumalo Stream	2012	800	320	770	6 (0.78)	250 (32.5)	94 (12.2)	18 (2.3)
	1999	1000	189	710	0 (0)	317 (44.6)	230 (32.4)	27 (3.8)
`Āwehi Stream	2012	640	543	524	4 (0.76)	274 (52.3)	98 (18.7)	37 (5.2)
	1999	1000	734	734	0 (0)	511 (69.6)	30 (4.1)	15 (2.0)

[1] *Macrocyclops albidus, Acanthocyclops vernalis;* [2] *Megalagrion calliphya, Megalagrion blackburni, Anax strenuus*

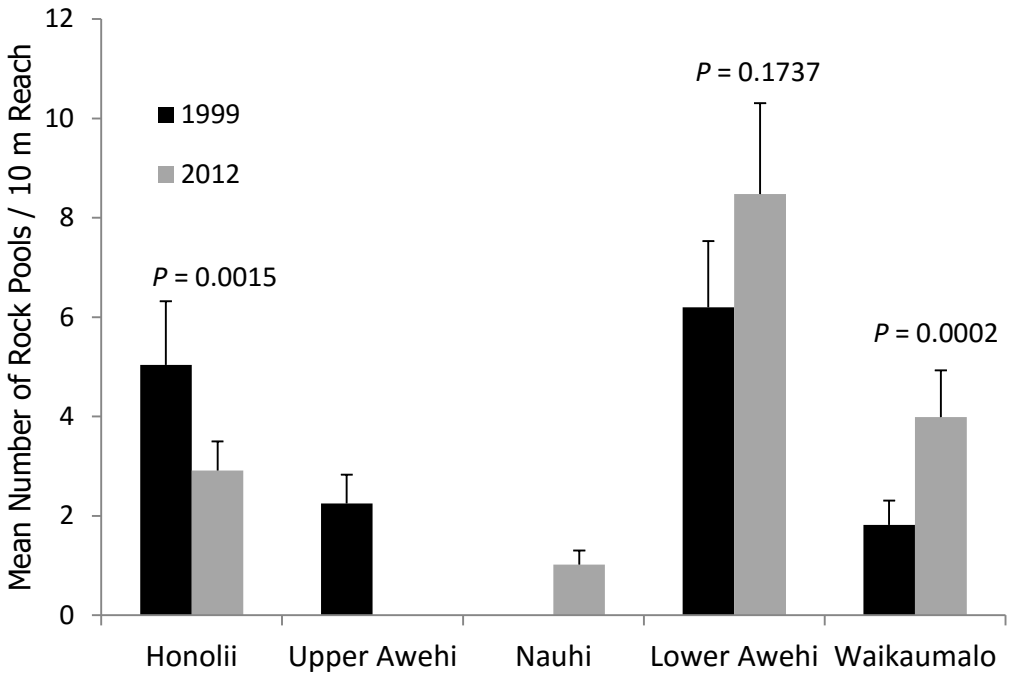

Figure 6. Mean number of rock pools in streams at Hakalau Forest NWR sampled in October 1999 and in October 2012. (means, 95% confidence intervals and P values of paired T tests).

Table 9. Precipitation and streamflow associated with stream surveys at Hakalau Forest NWR.

Stream	Year	Prior Rainfall Totals (inches)[a]			Discharge (cubic feet per sec)[b]		
		7 Days	14 Days	31 Days	0 Days	1 Day	2 Days
`Āwehi	1999	0.66	4.24	11.55	22	23	25
	2012	3.54	7.16	14.19	41	40	47
Waikaumalo	1999	1.7	3.06	9.15	56	20	16
	2012	0.04	2.75	7.14	23	17	18
Honoli`i	1999	4.2	4.69	7.82	23	32	59
	2012	0.81	1.73	14.96	121	17	12
Nāuhi	1999	-	-	-	-	-	-
	2012	1.06	4.07	13.17	-	-	-

[a]Ka`uku 140.5 HI, US (GHCND: USC00513473) 1999; Honomu 2.8 SW, HI US (GHCND: US1HIHI0014) 2012

[b]Honoli`i, USGS Gaging Station #16717000. Discharge on the day of survey (0 Days) and 1 and 2 days before survey.

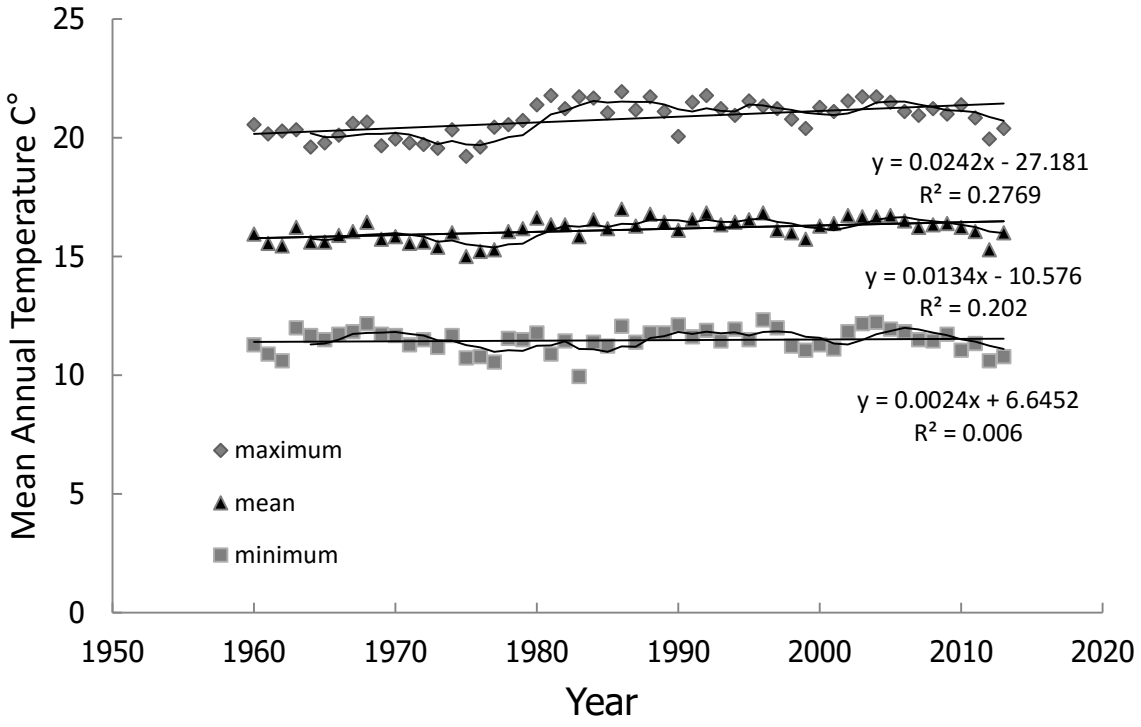

Figure 7. Fifty-three year linear and 5-year moving average trends in the mean annual temperatures recorded at 1,210 m asl in Hawai`i Volcanoes National Park, Headquarters.

DISCUSSION

Local Transmission of Avian Malaria at High Elevation

Unlike other studies in Hawai`i (van Riper et al. 1986, Freed et al. 2005, Atkinson and Samuel 2010), we did not find evidence of active transmission such as moribund or dead birds, changes in the infection status among recaptured resident birds or acute parasitemia in resident birds. Only one bird, an `i`iwi, had the diagnostic profile (high parasitemia and no detectable immune response) of an acute infection, however, `i`iwi are a vagile species that may acquire infections at lower elevations during daily or seasonal flights (Kuntz 2008). A few resident native birds at Nāuhi, Hawai`i `amakihi and `ōma`o, tested positive for malaria by PCR but were negative by immunoblot. These presumptive acute infections could not be confirmed by microscopy but may represent very early infections before parasitemias are detectable in blood smears and before immune reactions are initiated. Evidence of active transmission, like acute parasitemia or clinical signs, has been scarce at HFNWR. In 15 years of research at upper Pua `Ākala (1,900 m asl), Freed and coworkers, observed only two cases of locally-acquired, acute infection; once in 1995 when a brood of Kalij pheasant *Lophura leucomelanos* chicks tested positive by PCR (Freed and Cann 2013b) and again in 2001 when a moribund Hawai`i `ākepa presented with vague malarial symptoms (Freed et al. 2005). The Hawai`i `ākepa infection was never confirmed, but the overall prevalence at Pua `Ākala doubled at that time (Freed et al. 2005). While we did not observe or confirm local, acute infections, we did detect chronic

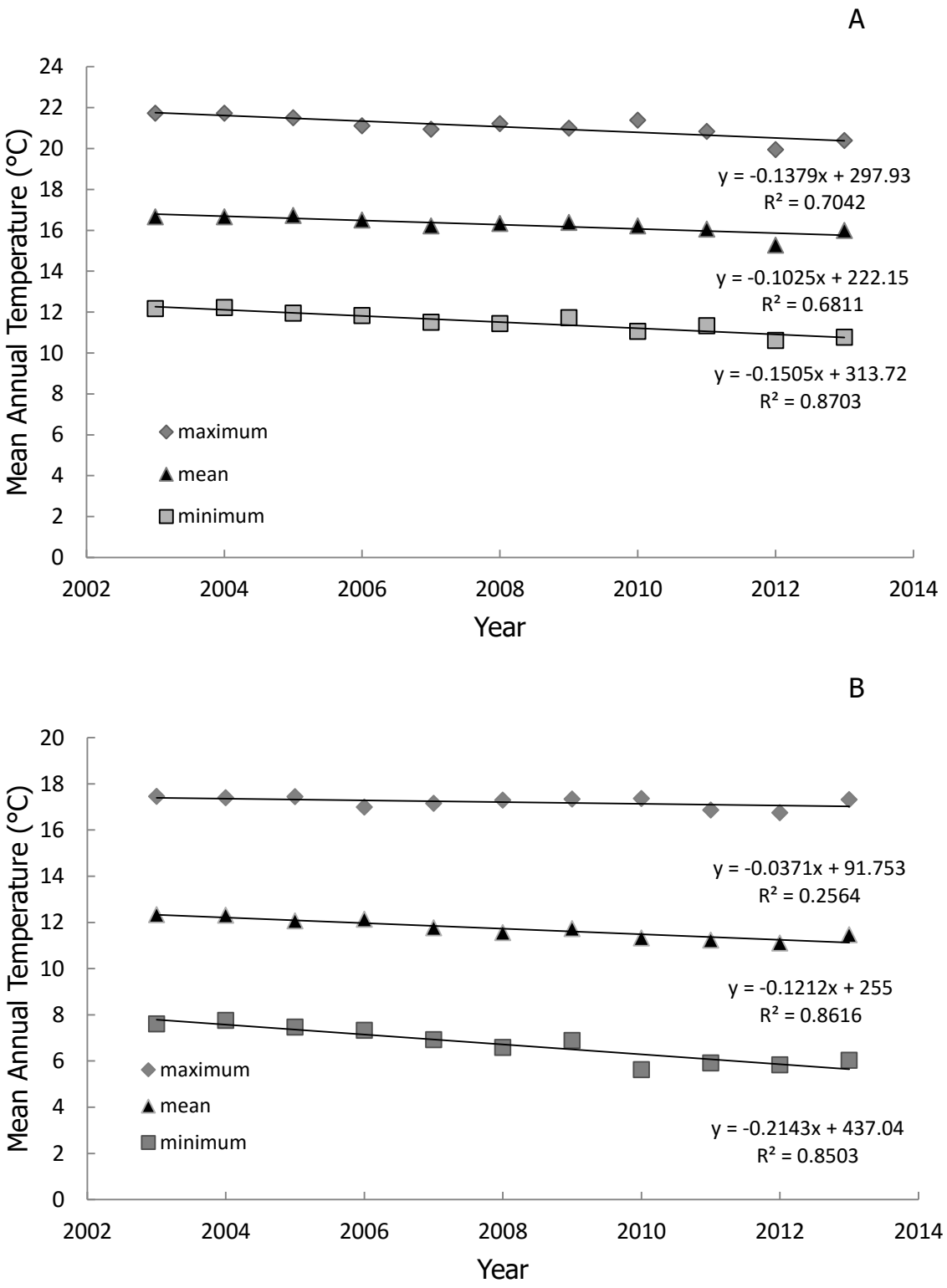

Figure 8. Ten-year, linear trend in the mean annual temperatures recorded at A) 1,210 m asl in Hawai`i Volcanoes National Park, Headquarters and B) 1,950 m asl in Hakalau Forest NWR.

infections in three, resident, native species (Hawai`i `amakihi, `ōma`o, and Hawai`i `elepaio) from which we can infer past, local transmission. These observations, and earlier estimates of prevalence at upper Pua `Ākala (2.1%, 1,900 m asl) and upper Maulua (2.6%, 1,800 m asl) (Feldman et al. 1995), suggest that that transmission of avian malaria in high elevation forests at HFNWR may be a relatively rare event particularly during the bird breeding season when the majority of our sampling took place.

Among the three resident species at Nāuhi, `ōma`o had the highest prevalence of avian malaria and, in 1998 and 2012, we found AHY `ōma`o with chronic infections at both lower Pua `Ākala and Nāuhi indicating that some level of transmission had occurred within the last year. `Ōma`o have a high tolerance to avian malaria and mid elevation populations on Mauna Loa have a high prevalence of chronic infection (Atkinson et al. 2001). Since `ōma`o are considered highly sedentary and are not known to disperse far from their natal territories (Ralph and Fancy 1994, Wakelee and Fancy 1999, Wu et al. 2014) these chronic infections provide the best evidence for local transmission at Nāuhi. We also found infected Hawai`i `elepaio at Nāuhi and Maulua in 1998. Adult Hawai`i `elepaio are also sedentary and young birds disperse less than 600 m from their natal territory (vanderWerf 1998, 2008). Like `ōma`o, infections in Hawai`i `elepaio are likely acquired locally. Freed et al. (2005, 2013) found Hawai`i `elepaio to have the highest prevalence (> 50%) among resident forest birds at upper Pua `Ākala and identified two local epizootics in 1995 and 2001 based largely on increases in Hawai`i `elepaio prevalence (Freed and Cann 2013). However, at Nāuhi in 1998–1999 we found Hawai`i `elepaio to have a lower prevalence of avian malaria (7%) than `ōma`o (13%) or Hawai`i `amakihi (12%) and in 2012, we did not detect avian malaria in the 36 Hawai`i `elepaio sampled from our three sites. Freed and Cann (2013b) suggested that Hawai`i `elepaio may be a key disease reservoir in high elevation forests given the observed prevalence of avian malaria and the nesting and roosting height of this species. Based on their relative abundance and the observed prevalence during this study, it seems more likely that `apapane, Hawai`i `amakihi and `ōma`o are more important reservoirs of avian malaria in the high elevation forests in HFNWR. Hawai`i `amakihi was the second most abundant bird captured during our study. Hawai`i `amakihi are considered non-migratory and population genetics studies on Mauna Loa suggest little movement between altitudinal populations (Foster et al. 2007, Eggert et al. 2008). Although some researchers have observed seasonal, altitudinal movement in what appears to be a response to decreased food availability (Lindsey et al. 1998), we did not observe a decrease in capture rates following the breeding season and assume that Hawai`i `amakihi were year-round residents of the study grid and had locally acquired infections as well.

Although the chronic infections of resident species at Nāuhi indicate past local transmission, we were unable to detect the vector, *C. quinquefasciatus* despite trapping efforts spread out across the year. We found *C. quinquefasciatus* to be exceedingly rare at HFNWR having detected only one adult at lower Pua `Ākala in 382 trap-nights and no larvae in over 2,400 inspections of potential larval mosquito habitat. Infrequent mosquito surveys at HFNWR in the past have detected few *C. quinquefasciatus* (LaPointe 2000). Swezey and Williams (1932) reported observing *C. quinquefasciatus* larvae in a spring pool at Nāuhi in 1931. In 1993, CO_2 and gravid traps were operated for a total of 697 trap-nights over a nine-month period at Pua `Ākala (1,900 m asl) and upper Maulua (1,500–1,800 m asl). Twenty-eight *C. quinquefasciatus* were captured at upper Maulua (1,500–1,800 m asl) but no mosquitoes were captured from Pua `Ākala. None of the mosquitoes from Maulua were infected with *P. relictum* (LaPointe 2000). Larvae were observed in old cattle troughs and old tires surrounding the plastic liner of a catchment pond in the former high pasture at upper Maulua. These man-made larval mosquito

habitats were quickly removed from the refuge (LaPointe 2000). Between 1994 and 1997 aquatic habitats were sampled throughout the year at Pua `Ākala, Nāuhi, and Maulua (1,500–1,700 m asl) but no mosquito larvae were found in over 1,000 observations (Woodworth et al. 2001). Freed et al. (2005) set out infusion-baited buckets at Pua `Ākala (1,900 m asl) from 2001 to 2004 to attract ovipositing *C. quinquefasciatus.* In the equivalent of 6,816 trap-nights only one egg raft was collected. Although *C. quinquefasciatus* adults and immature stages have been found as high as 1,981 m asl (Pohakuloa) in association with man-made habitat (Komatsu 1966, LaPointe 2000), there are no records in Hawai`i of permanent populations in natural lands above 1,500 m. Climate-based modeling of the altitudinal limit (1,472 m asl) for permanent populations of *C. quinquefasciatus* supports these field observations (Ahumada et al. 2004). However, the model also predicts that during the warmer summer months, short-lived populations founded by dispersed individuals may exist at elevations as high as 1,715 m asl. The source of these transient populations may provide an alternative explanation. Many researchers have noted that in some montane areas *C. quinquefasciatus* was only found following certain prevailing winds such as weakening trade winds and Kona storms (Goff and van Riper 1981, Scott et al. 1986). Freed and Cann (2013b) postulated that climate warming drives an increase in wind-dispersed, infected mosquitoes from lower elevations. The Enhanced-Mosquito-Movement (EMM) model provides a theoretical mechanism to account for transmission at high elevations where the near-complete absence of mosquitoes and cool temperatures would preclude local transmission (Freed and Cann 2013b).

Spatial Variation in Avian Malaria Prevalence

This study also documented spatial variation of avian malaria prevalence in forest bird communities at HFNWR. We found that the overall prevalence of avian malaria at each of our sites exceeded the 2–5% prevalence previously reported at upper Pua `Ākala (1,900 m asl; Feldman et al. 1995, Freed et al. 2005, Freed and Cann 2013b). During our first sampling period (1998–1999), the prevalence of avian malaria was two times greater at Nāuhi (10%; 1,600 m asl) and 3–4 times greater at the lower Pua `Ākala and Maulua sites (15–20%; 1,300 m asl), respectively, than the estimated prevalence at upper Pua `Ākala in 2001 (Freed et al. 2005). This inverse relationship between elevation and prevalence results in a two-fold reduction in malaria prevalence with every 300 m gain in elevation. Similar altitudinal gradients in prevalence have been documented in the Hawaiian Islands (van Riper et al. 1986, Atkinson et al. 2005, LaPointe et al. 2012) though prevalence at higher elevations varies considerably from 0% at 1,420 m at Kipahulu Valley, Haleakalā National Park, Maui (Aruch et al. 2007) to 13% at 1,830 m in the Kona Forest Unit of HFNWR on leeward Mauna Loa (Atkinson et al. 2005). This altitudinal trend in prevalence has been attributed, at least in part, to climatic constraints imposed on vector populations and parasite development (LaPointe 2000, Ahumada et al. 2004, LaPointe et al. 2010). As mentioned earlier, climate limits the altitudinal distribution of *C. quinquefasciatus* but temperature also has a profound effect on the sporogonic development of the parasite. In fact, at upper Pua `Ākala (1,900 m asl) the mean temperature falls just below the threshold temperature for *P. relictum* development (13°C; LaPointe et al. 2010, Samuel et al. 2011). Despite this low mean temperature, some parasite development can occur during midday hours when temperatures rise above this threshold (Paaijmans et al. 2009) and infections in resident birds at upper Pua `Ākala have been attributed to exceptional warm summer temperatures (Freed et al. 2005). However, at 1,900 m, even with daily maxima reaching 5°C above the mean, the slow accrual of the 86 day-degrees necessary to complete sporogony may not be reached during the lifespan of the mosquito (LaPointe et al. 2010).

Within climatic constraints, site-to-site variability in prevalence is further influenced by land use, forest fragmentation, habitat disturbance and the occurrence of streams and natural wetlands as it relates to larval mosquito habitat availability and bird/vector movements (Reiter et al. 2007, Kuntz 2008, LaPointe 2008, LaPointe et al. 2009). We also observed differences in prevalence between our two lower elevation sites during both the 1998–1999 and 2012 surveys. Prevalence of avian malaria was higher in lower Maulua than in lower Pua `Ākala, although these differences were not statistically significant. Similarly, Feldman et al. (1995) found no significant differences in overall prevalence of avian malaria between higher elevation sites at Pua `Ākala and Maulua. This was unexpected since a clear gradient of increasing forest fragmentation and habitat disturbance exists along North/South and altitudinal gradients within HFNWR. The Pua `Ākala tract remains relatively intact but the forest becomes increasingly fragmented moving north to Nāuhi and Maulua areas; which were impacted by historical cattle ranching. Habitat disturbance, primarily by feral pigs, remains high in the unmanaged lower elevations but varies in the managed upland tracts from minimal disturbance at Pua `Ākala to increasing levels at the northern end of the refuge (Hess et al. 2006, 2013). In this study, we observed a clear relationship between feral pig activity and the amount of feral pig-created larval mosquito habitat. While we did not find *C. quinquefasciatus* larvae at HFNWR, tree fern cavities and wallows are the main habitats used by larval *C. quinquefasciatus* in nearby Laupāhoehoe and Waiākea Forest Reserves (LaPointe 2000, LaPointe et al. 2009, LaPointe unpublished data).

Temporal Variation in Avian Malaria Prevalence and Impacts of Climate Change

Freed and Cann (2013) reported an increase in avian malaria prevalence in the forest bird community at upper Pua `Ākala from 1988 to 2002 and attributed this to the upslope movement of infected mosquitoes. We also found a secular trend in avian malaria prevalence over a 14-year period from 1998 to 2012, however, unlike Freed and Cann (2013), we found a significant decrease in prevalence at all our study sites. Avian malaria prevalence had decreased by half at our Nāuhi and lower Maulua sites. At lower Pua `Ākala, we found no infected birds in 2012 although the sample size was small (N = 34). Furthermore, with the exception of `ōma`o, all native species saw a similar reduction in malaria prevalence including `apapane and `i`iwi which suggests that factors limiting transmission at high elevation were also operating at lower elevations and possibly across the broader, resource landscape use by these vagile species (Kuntz 2008). Why the disparity in results? The earlier increase in avian malaria reported by Freed et al. (2005, 2013) was driven largely by a magnitude-scaled increase in the prevalence of one species, the Hawai`i `elepaio. However, little to no increase was seen in the prevalence of other resident, native species like Hawai`i `amakihi and `ōma`o (Freed et al. 2005). If transmission has increased at Pua `Ākala, then an increase in prevalence should be seen in these malaria-susceptible species (Atkinson et al. 2000, Atkinson et al. 2001b) as well, assuming equal attractiveness and availability to mosquitoes. Freed and Cann (2013) suggested that Hawai`i`elepaio may be encountered more often by host seeking mosquitoes due to their nesting/roosting sites but `ōma`o nest and roost at the same height (vanderWerf 1998, Wakelee and Fancy 1999). An alternative explanation to differential transmission is sampling bias that does not account for the cumulative effects of chronic infections. Hawai`i `elepaio are an exceptionally long-lived and malaria tolerant species (vanderWerf 2004, 2008). As such, a population cohort will accumulate chronic infections over time. If sample sizes are small and/or strongly age-biased, then inaccurate prevalence estimates are likely. In a population where prevalence is age dependent, a prevalence estimate based on a small sample of disproportionately older birds could appear as a profound change from an earlier estimate based on a sample of younger birds. This would be more pronounced if malaria transmission

were episodic (every few years) rather than seasonal (annual). In the present study, all Hawai`i `elepaio were SY or older birds and age ratios were similar between sampling periods.

Experimental and modeling studies have suggested that climate change, in particular a warming trend, will enhance transmission of avian malaria in the Hawaiian Islands by releasing vectors and the parasite from thermal constraints (Benning et al. 2000, Atkinson and LaPointe 2009, LaPointe et al. 2010, Samuel et al. 2011). Over the last decade, the prevalence of avian malaria has increased dramatically in forest birds inhabiting the Alakai Plateau in Kaua`i (Atkinson et al. 2014). Although mean daily temperature in the Alakai Plateau is increasing 0.2°C per decade, the evidence from Kaua`i suggests that changes in precipitation regime may have a more direct effect on vector abundance than increasing temperature (Atkinson et al. 2014). On the island of Hawai`i, Freed et al. (2005, 2013b) have suggested that increases in mean daily temperature have already enhanced transmission at HFNWR. They proposed that while temperatures at 1,900 m may still be inadequate for on-site completion of sporogony, warming at lower elevations will increase the number of infective mosquitoes that can disperse upslope. In support of this theory, we documented a warming trend (0.134°C/decade) at mid-elevation in long-term data collected in nearby Hawai`i Volcanoes National Park (1,210 m). Despite this and evidence of a general warming throughout the Hawaiian Islands (Giambelluca et al. 2008), we did not observe an increase in prevalence in the last 14 years. Modeling avian malaria transmission and climate change in Hawai`i, Liao et al. (2015) found that no significant impacts at mid and high elevation could be expected for the next 25 years. These predictions, however, are based on global warming models that had not been locally downscaled for the Hawaiian Islands. Our examination of long term temperature data from the island of Hawai`i revealed apparent cycles in mean annual temperature and a decreasing trend (-1°C/ decade) over the last decade (2001–2013) at both Hawai`i Volcanoes National Park and upper Pua `Ākala. The 1°C decrease in mean annual temperature probably had little effect on transmission at mid-elevation where seasonal transmission is already efficient (Samuel et al. 2011) or at upper Pua `Ākala, where the likelihood of sporogonic development completion is marginal.

Models defining the general drying trend in precipitation for the Hawaiian Islands may be more accurate and significant to future predictions of malaria transmission at HFNWR (Chu and Chen 2005, Timm and Diaz 2009). A slightly drier future with shifts in seasonal precipitation could enhance transmission (Atkinson et al. 2014, Liao et al. 2015) but extended drought would have a profoundly opposite effect. With abnormally dry conditions preceding our survey for several years, larval mosquito habitat would become very limited and small, forest populations of *C. quinquefasciatus* may have died out, effectively contracting its range to the lowlands and more persistent larval habitat associated with agriculture and human residence. Populations of *A. j. japonicus*, with drought resistant eggs, would be able to persist or rebound rapidly. More recent dynamic downscaling of regional climate in the Hawaiian Islands (Zhang et al. 2012) have led to model predictions of wetter conditions along the Hamakua Coast. In general, wetter conditions will favor both feral pig and mosquito populations outside the managed areas at HFNWR.

Avian Pox at Hakalau Forest National Wildlife Refuge

Unlike avian malaria, we do not have a simple diagnostic test for *Avipoxvirus*. Without biopsied tissue from a suspect lesion we could not confirm our presumptive field diagnosis. Swollen, wet and scabbed over lesions and missing/deformed digits were all considered pox-like lesions in this study but may have resulted from injury, infection by other pathogens or a combination of these factors. For this reason, our prevalence estimates may be inflated. Still, van Riper et al. (2002) found the descriptive criteria for pox lesions to be 90% accurate when compared with

histological examination and/or virus cultivation in chicken egg, chorioallantoic membrane (CAM). The overall prevalence of pox-like lesions at HFNWR was similar to that of the forest bird community sampled in Kona Unit of HFNWR on leeward Mauna Loa (5%; Atkinson et al. 2005) but lower than the prevalence observed in windward Mauna Loa (13.5%; van Riper et al. 2002). As with earlier studies, we found pox-like lesions to be more prevalent in native birds than non-native birds (van Riper et al. 2002, Atkinson et al. 2005) but unlike these studies we did not find pox to be more prevalent among birds found positive for avian malaria (van Riper et al. 1986, Atkinson et al. 2005). When compared with sites on Mauna Loa, the prevalence of pox-like lesions was lowest at HFNWR in all native species. The low prevalence of pox-like lesions at HFNWR mirrors the low avian malaria prevalence and is consistent with the general absence of the mechanical vector, *C. quinquefasciatus.* However, unlike avian malaria, we did not see an increase in the prevalence of presumptive pox at lower elevation or a decrease in prevalence over the study period. A low, consistent prevalence of presumptive pox at Nāuhi suggests that some pox transmission may occur in the absence of *C. quinquefasciatus*. One possible explanation could be the presence of an alternative vector, such as *A. j. japonicus*, that could be a competent mechanical vector of *Avipoxvirus* but is incapable of vectoring avian malaria. While the timeframe of *A. j. japonicus* introduction fits this scenario, we did not observe this species at Nāuhi during our study. While the prevalence of pox-like lesions was low during our surveys, *Avipoxvirus* epizootics have been documented in the past at HFNWR (vanderWerf 2001). VanderWerf (2001) observed a high prevalence (40%) of healed pox-like lesions in Hawai`i `elepaio at Maulua (1,500 m asl) in 1994. Prevalence within different aged individuals suggested an epizootic had occurred in 1992. In 1994, the density of Hawai`i `elepaio at Maulua was 49% lower than Pua `Ākala but the population of Hawai`i `elepaio rebounded in the four years following the epizootic. During this time, vanderWerf (2001) did not observe active pox in Hawai`i `elepaio at either study site and found annual survival and reproductive success to be unaffected by past pox infection.

Changing Patterns of Available Larval Mosquito Habitat

Cryptic populations of *C. quinquefasciatus* have been difficult to detect at HFNWR yet local transmission in resident birds still occurs at high elevations. Whether mosquitoes develop on site (Ahumada et al. 2004) or disperse up slope from warmer elevations (Freed and Cann 2013b) the relative abundance of vectors depends on available larval habitat. Vector abundance will, in turn, have a direct effect on transmission rate and therefore prevalence. Since feral pig-created, larval mosquito habitat is the most productive habitat type occurring in natural areas elsewhere on Hawai`i Island (LaPointe et al. 2012) we assume this habitat is important at HFNWR as well. Refuge efforts to eliminate feral pigs from fenced enclosures in the upper half of HFNWR began in 1989 and have been generally successful at reducing the number of pigs and subsequent habitat disturbance (Hess et al. 2006, Hess et al. 2013). We observed significant change in feral pig activity and the density of tree fern cavities at three sites which reflects both the efficacy of pig removal efforts and the impact of environmental factors. In Nāuhi, we observed a decrease in the frequency of recent pig sign between sampling periods, while at upper Pua `Ākala pig sign increased in frequency. Both sites were actively managed over the years but a reduction in refuge staff and breaches in the fence line limited removal effort hours and resulted in apparent population gains. In 1998, upper Pua `Ākala was essentially pig-free with no recent sign recorded from the site but, by 2012 pigs had made incursions into upper Pua `Ākala creating suitable larval habitat for *C. quinquefasciatus*. The recent surveys at Nāuhi, however, suggest that recent pig removal efforts have made an impact in reducing feral pig abundance (Hess et al. 2013).

The most unexpected observation was the dramatic decreases in recent feral pig sign and densities of tree fern cavities in the unmanaged lower Pua `Ākala and Maulua sites. These significant decreases in pig activity suggest a movement of pigs away from interior forests of HFNWR or some environmental regulation of pig numbers. Additional feral pig survey work in lower HFNWR by U.S. Geological Survey and refuge biologists has also documented a significant drop in pig abundance between 2010 and 2014 (Leopold et al. 2015). On Hawai`i Island, Giffin (1978) observed that feral pigs moved greater distances in response to free water availability and that feral pig abundance was greatest in wet, montane rainforests. Studies in Texas have also demonstrated a positive correlation between feral pig abundance and precipitation (Adkins and Herveson 2007). Although studies of feral pig population dynamics are typically limited, monitoring by feral pig activity, damage, and game record indices indicates that feral pig abundance plummets during prolonged drought in California (Peart et al. 1994). Along with the loss of free water, drought will reduce the availability of green browse, fruits, and invertebrates — all main components of the feral pig diet. Mean annual precipitation in the Hawaiian Islands has been below the long-term average (> 50 years) since 1990 (Diaz et al. 2005) and on Hawai`i Island annual precipitation totals have been below average since 2008 (Figure 9). In 2010 windward Hawai`i received 50% of the mean annual rainfall; the driest year since 1950. This extended drought likely had an impact on feral pig abundance. If this general drying trend extends into the future (Timm and Diaz 2009), a drier environment at HFNWR may result in direct and indirect change in available larval mosquito habitat.

Indirectly, drier conditions may lead to lower feral pig abundance in unmanaged sections of the refuge and fewer pigs should relate to fewer tree fern cavities and wallows. Directly, decreased precipitation may reduce streamflow in the lower half of HFNWR thereby increasing the number of rock pools along streambeds available to larval mosquitoes and/or the interval between scouring spates (Atkinson et al. 2014, Strauch et al. 2015). Evidence of this effect is seen in an increased abundance of rock pools along the lower `Āwehi and Waikaumalo Streams in 2012. Interestingly, the reverse pattern was seen at the upper reaches of Honoli`i Stream where streamflow is intermittent and rock pools probably dry up between precipitation events.

Biotic Resistance to *Culex quinquefasciatus*

Culex quinquefasciatus is widely distributed and seasonally abundant in wet and mesic forests on Hawai`i Island (LaPointe 2000, LaPointe et al. 2009, 2012). Therefore, the near complete absence of *C. quinquefasciatus* in HFNWR, especially at the lower elevation forests where larval habitats are abundant, was very unexpected. Isolation from residential and agricultural areas where large populations of mosquitoes are sourced may be part of the explanation but biotic resistance by native predators and invasive competitors may also play a role in limiting *C. quinquefasciatus* at HFNWR. Although we did not detect *C. quinquefasciatus* larvae during our surveys, we did observe larvae of *A. j. japonicus* in tree fern cavities and rock pools in 2012. *Aedes japonicus japonicus* is a highly invasive mosquito, native to temperate Asia, but now established throughout the eastern United States and parts of Europe (Kaufman and Fonseca 2014). Larvae are found in rock pools and the various types of natural and artificial containers commonly inhabited by *C. quinquefasciatus* (Kampen and Werner 2014). This mosquito was first reported from Hawai`i Island in 2004 and has rapidly spread island-wide (Larish and Savage 2005, Larish et al. 2010). In the Laupāhoehoe and `Ōla`a Forest Reserves, *A. j. japonicus* and *C. quinquefasciatus* co-occur in tree fern cavities though species dominance varies with season (LaPointe unpublished data). While no clear evidence exists for competitive displacement of *Culex* spp. by *A. j. japonicus*, researchers in northeastern United States have reported a decline in the distribution and abundance of native mosquito species, including *Culex*

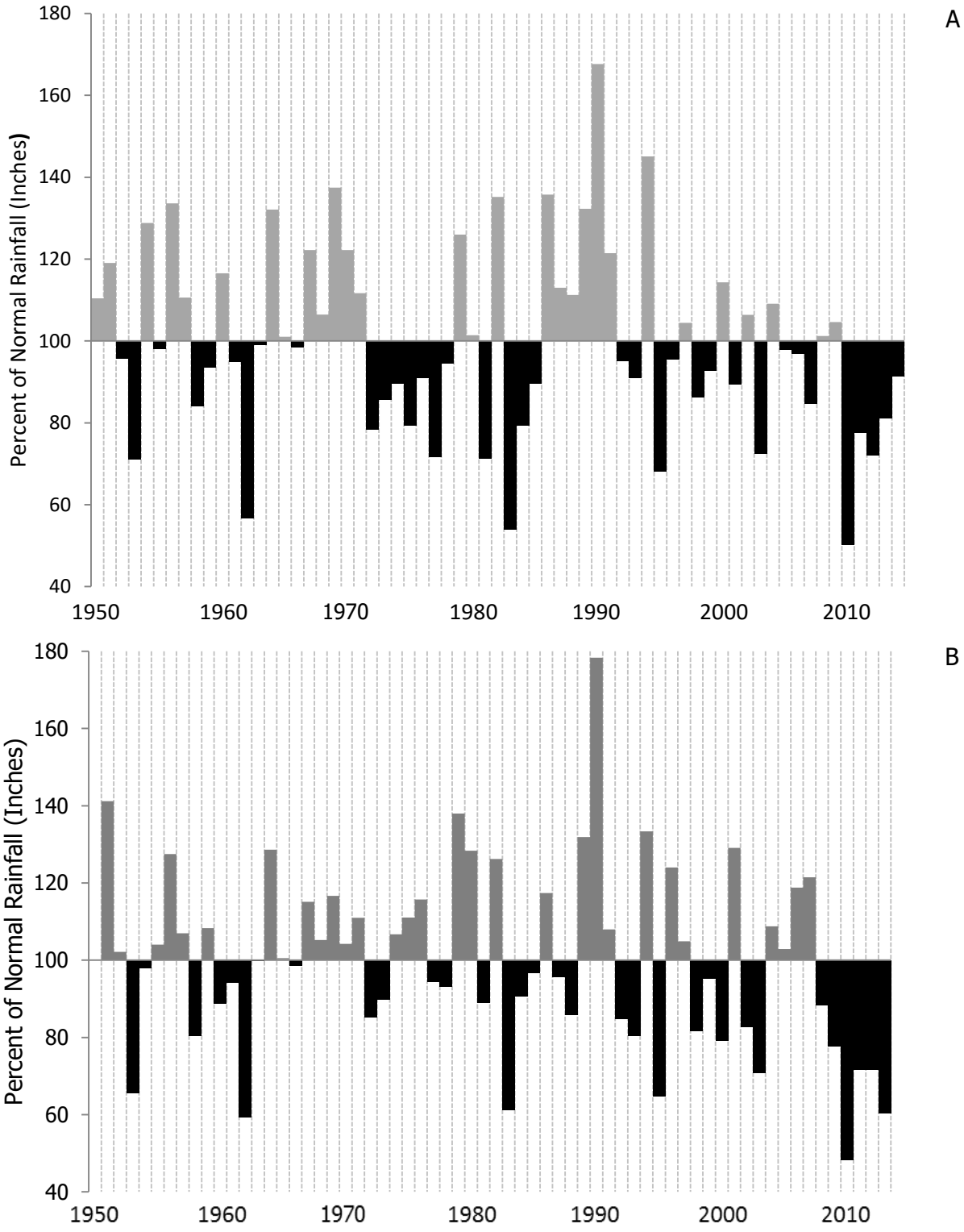

Figure 9. Total annual precipitation time series from 1950–2014. Values are departures from mean. A) Hilo International Airport, 27 m asl and B) Hawai`i Volcanoes National Park, Headquarters, 1,210 m asl.

restuans, since the local establishment of *A. j. japonicus* (Andreadis and Wolfe 2010). Environmental factors may be critical to competitive outcomes and since *A. j. japonicus* is temperate mosquito it may have a competitive edge over *C. quinquefasciatus* at cooler, higher elevations in Hawai`i. Similarly, the drought resistant eggs of *A. j. japonicus* may be a selective advantage if the climate continues to get drier. Since *A. j. japonicus* is not a competent vector of *P. relictum* (LaPointe unpublished data), any competitive displacement of *C. quinquefasciatus* populations by *A. j. japonicus* could have a profound effect on transmission.

Aside from interspecific competition with *A. j. japonicus*, a suite of endemic and indigenous aquatic predators may exert biotic resistance on *C. quinquefasciatus* inhabiting rock pools. The endemic veliid, *Microvelia vagans* is a ubiquitous water strider found in rock pools and ground pools in Hawai`i where it has been observed feeding on emerging chironomid flies (Williams 1943). While we have no direct evidence of *M. vagans* predating on *C. quinquefasciatus* in the field, we have observed *Microvelia vagans* feeding readily on emerging *C. quinquefasciatus* in the laboratory (LaPointe unpublished data). Similar laboratory observations have been reported for *Microvelia cavicola* feeding on emerging *Culex urichii* in Panama (Yanoviak 1999). Early instar larvae in rock pools may be faced with intense predation by cyclopoid copepods (Marten et al. 1994; 2000). *Acanthocyclops vernalis* and *Macrocyclops albidus* were common in HFNWR stream rock pools and have repeatedly proven to be effective predators of *C. quinquefasciatus* larvae in laboratory trials (Marten and Reid 2007) and in nature (Marten et al. 2000). Latter instar larvae are prey for endemic *Anax strenuus* dragonflies and *Megalagrion* damselflies (Williams 1936, Hobbelen et al. 2013). *Megalagrion calliphya* were the most common odonate we encountered in perched rock pools and modeling efforts to examine predator prey dynamics suggest that *M. calliphya* may regulate *C. quinquefasciatus* populations under certain climate regimes (Hobbelen et al. 2013). This small assemblage of aquatic predators is a formidable gauntlet to *C. quinquefasciatus* throughout its life cycle and healthy streams with thriving native predators may provide biotic resistance to limit mosquito populations even in the face of climate change.

Recent Threats to and Changes in the Avian Community at Hakalau Forest NWR.
In the last decade, Freed and coworkers (2005, 20012, 2013a,b, 2014) have reported population declines of most native species and collapse of endangered honeycreeper populations (Hawai`i `ākepa and Hawai`i creeper) at HFNWR. Although food resource competition with invasive Japanese white-eyes has been suggested as the ultimate cause for the population collapse of Hawai`i creeper (Freed and Cann 2012, 2013b, 2014), increasing prevalence of mosquito-borne disease (Freed et al. 2005, Freed and Cann 2013a) and explosive increase in ectoparasites (Freed et al. 2008) have been implicated as contributing factors in species population declines at HFNWR. These negative population trends, however, have not been confirmed by analysis of long-term monitoring data (Camp et al. 2010, Camp et al. 2015, Guillaumet et al. 2015) and our study found no evidence of an increased prevalence in mosquito-borne disease or ectoparasites that could potentially impact the health of birds. In fact, we found the prevalence of avian malaria to be lower than the prevalence observed 14 years earlier. Furthermore, we examined over 200 birds at HFNWR and found no parasitic skin mites (*Knemidokoptes* spp.) or feather degrading, chewing lice (Phthiraptera: Mallophaga) on native birds. Although feather mites (*Analges* sp. and *Proctophyllodes* sp.) were common on most native species, they are generally considered harmless commensals (Proctor 2003).

While we did not monitor bird densities over the course of our study, we did observe endangered honeycreepers, Hawai`i `ākepa, `akiapōlā`au and Hawai`i creeper, at our low

elevation (~1,300 m asl) sites in 2012 that were last detected in this area more than three decades ago (1977) during the Hawaii Forest Bird Survey and prior to the establishment of the refuge (Scott et al. 1986). During the 1977 surveys, the lowest detection of an `akiapōlā`au was made at 1,556 m asl. Interestingly, we did not observe endangered honeycreepers during our earlier sampling period in 1998–1999 even though those visits 1) occurred during the breeding season and late summer, 2) were longer in duration, 3) logged more mist-netting hours and 4) included individuals who were trained to recognize native birds by sight and sound. All three species are believed to be highly susceptible to avian malaria and pox virus and currently limited in distribution to the cooler, high elevations of the refuge. While the disparity in our observations of endangered honeycreepers during our study may simply reflect the rarity of these species, the potential return of these endangered honeycreepers to lower elevation forests at HFNWR may be a response to the observed decrease in avian malaria transmission at the refuge.

CONCLUSIONS, MANAGEMENT IMPLICATIONS AND FUTURE RESEARCH

Our study documented spatial trends and temporal changes in avian malaria prevalence, mosquito presence, larval mosquito habitat and feral pig activity at Hakalau Forest National Wildlife Refuge. We found evidence of limited local transmission in high elevation forest (Nāuhi, 1,700 m asl) within HFNWR, a general pattern of increasing prevalence at lower elevations and along a South to North gradient and, most importantly, an approximately two-fold decrease in the prevalence of avian malaria over the intervening 14 years. The decrease in avian malaria prevalence appears to be refuge-wide and may be reflected in our observation of endangered honeycreepers at our low elevation (~1,300 m asl) sites. While a decade of cooler temperatures may have contributed to lower transmission at high elevation, a general absence of the vector of avian malaria, *C. quinquefasciatus,* from our study sites would account for the low prevalence and disease trends. We observed a number of factors that might limit vector distribution and abundance on the refuge including a decrease in feral pig activity and the density of larval mosquito habitat created by pigs and biotic resistance from native predators and invasive competitors (*A. j. japonicus*).

While current predictions of climate change in the Hawaiian Islands include a gradual warming and enhanced transmission of avian malaria by mid-century (Liao et al. 2015), the current cooling trend at high elevation HFNWR over the last decade demonstrates the value of more frequent observations in the long-term monitoring of avian disease. Avian disease monitoring at HFNWR has been sporadic at best and conflicting reports of increasing or decreasing prevalence reflect differences 1) in diagnostic methodology, 2) sample size and composition (species and age) and 3) time frame under examination. Point prevalence estimates based on cross sectional surveys may not give an accurate estimate of change in disease risk or transmission. Future surveys for more accurate assessment of disease trends can be improved by focusing on AHY resident species to measure malarial incidence over the preceding six-month transmission period. Hawai`i `amakihi are good candidates for monitoring because of their relative abundance in the forest bird community and limited dispersal behavior. `Ōma`o and Hawai`i `elepaio should be considered, as well, due to their sedentary nature and site tenacity. Hawai`i `elepaio may be particularly well-suited for longitudinal studies when adequate numbers can be captured. The best estimates would be derived from data collected across multiple years to increase sample size and capture rare events. Monitoring of disease prevalence in resident birds could be incorporated into ongoing avian demography studies. The present rarity of mosquitoes

at HFNWR makes monitoring vectors for disease presence impractical. Oviposition buckets, however, could be used for effective detection of incipient populations. Buckets are inexpensive and require little effort to monitor and maintain although they should be inspected and maintained every other week to prevent development of intercepted egg rafts.

Current efforts to reforest and restore forest bird habitat at HFNWR through the out-planting of native plants and feral pig control can have beneficial effects for reducing mosquito-borne avian disease. Closed-canopy and thick understory forests restrict mosquito immigration from unmanaged lands outside of the refuge (LaPointe 2008) and fewer pigs mean less aquatic habitat for larval *C. quinquefasciatus*. Restored habitat and fewer pigs will also have a positive effect on stream habitats and the diversity and abundance native, aquatic invertebrates, thereby providing greater biotic resistance to invading *C. quinquefasciatus.* Extending feral pig control to lower sections of the refuge will help to decrease malarial transmission and reduce the immigration of infected mosquitoes to upper elevation forest (Freed and Cann 2013b). Although feral pig control will tend to limit available larval mosquito habitat, it is crucial that resource management and research activities at the refuge do not create larval mosquito habitat or cause thermal enhancement of the adult mosquito resting environment. New infrastructure related to water catchment and impoundment can be designed and maintained to prevent use by larval mosquitoes. Structures, particularly those that absorb radiant energy, can also be designed to prevent adult mosquito access to these potentially warmer microhabitats.

Two main research questions remain regarding avian malaria transmission at HFNWR. First, how do resident species get infected at high elevation in the apparent absence of vectors and second, why hasn't *C. quinquefasciatus* become permanently established at HFNWR? Additional information on the altitudinal movement of resident birds and mosquitoes, finer scaled climate monitoring across HFNWR, and a better understanding of the impacts of competing mosquito species and invertebrate predators on larval, *C. quinquefasciatus* populations at HFNWR would help to address these questions.

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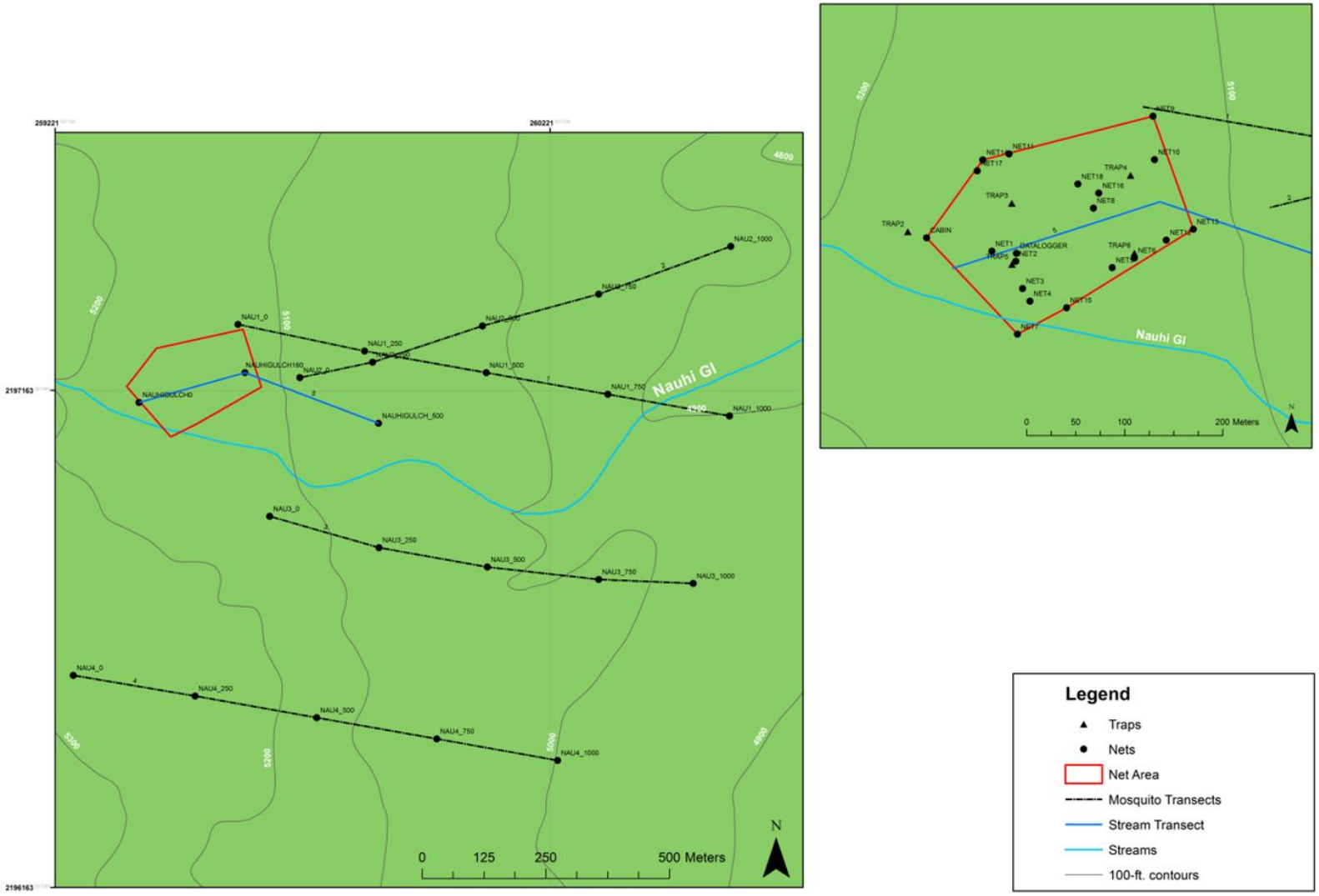
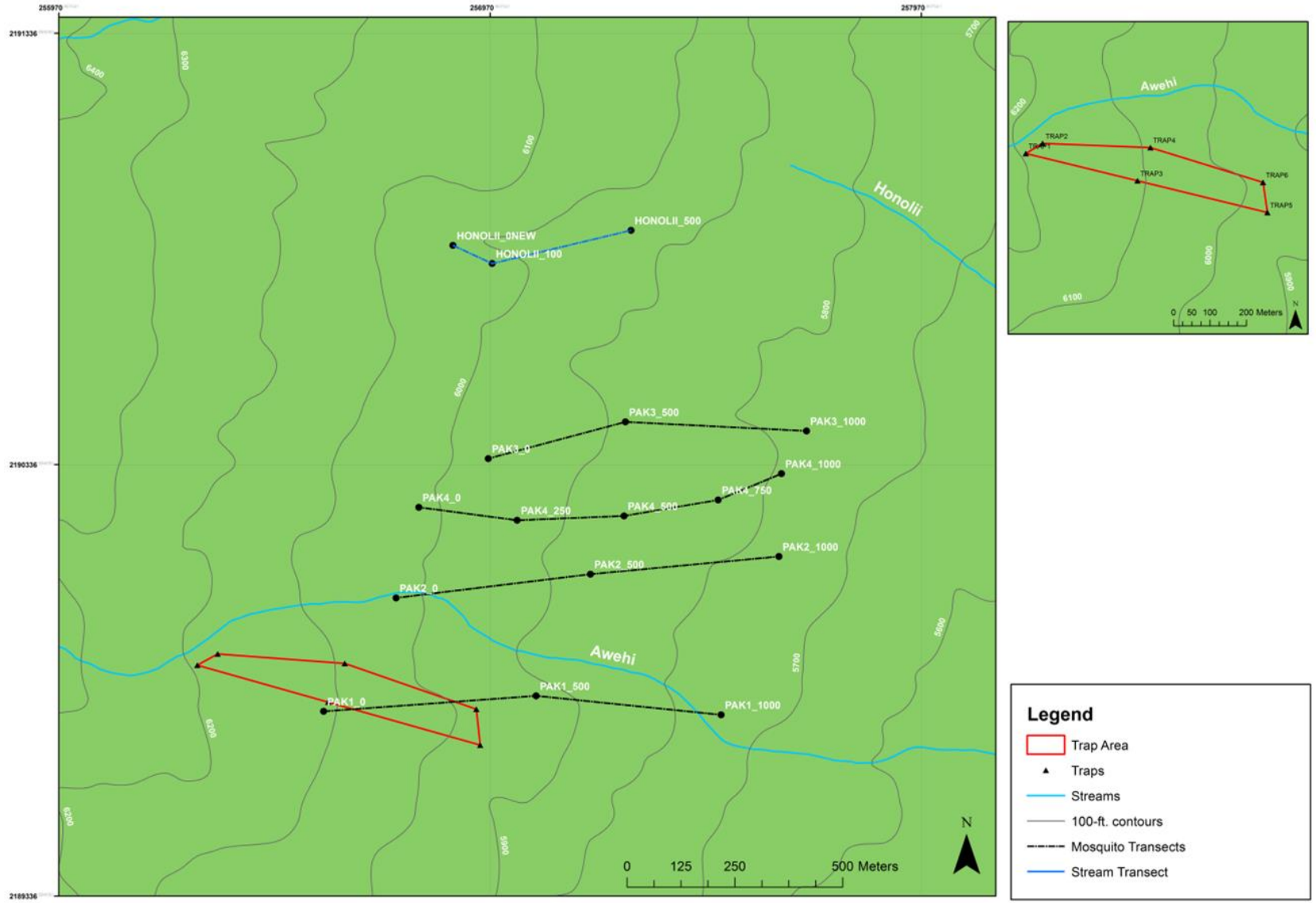
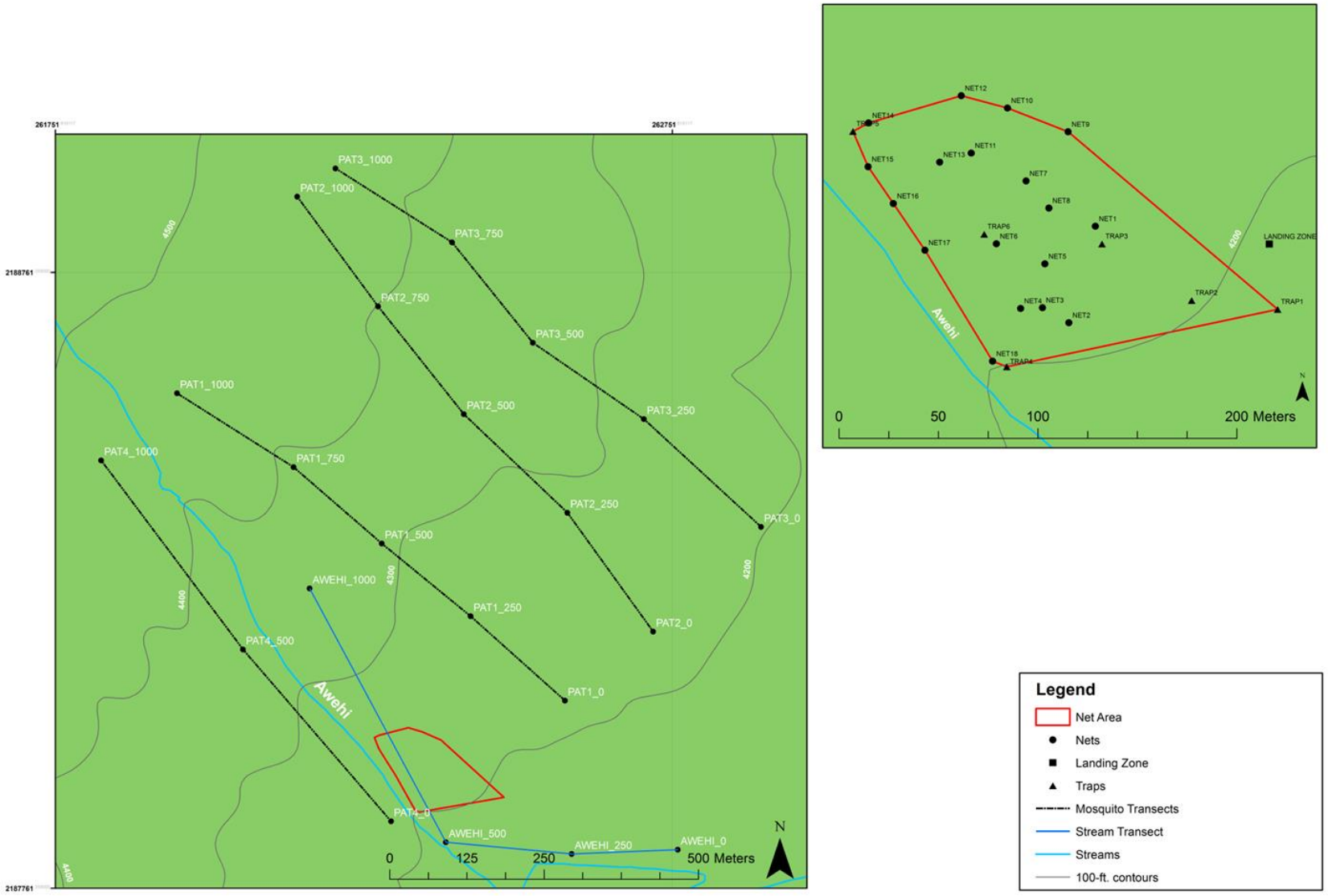
APPENDIX I SUPPLEMENTAL STUDY SITE MAPS



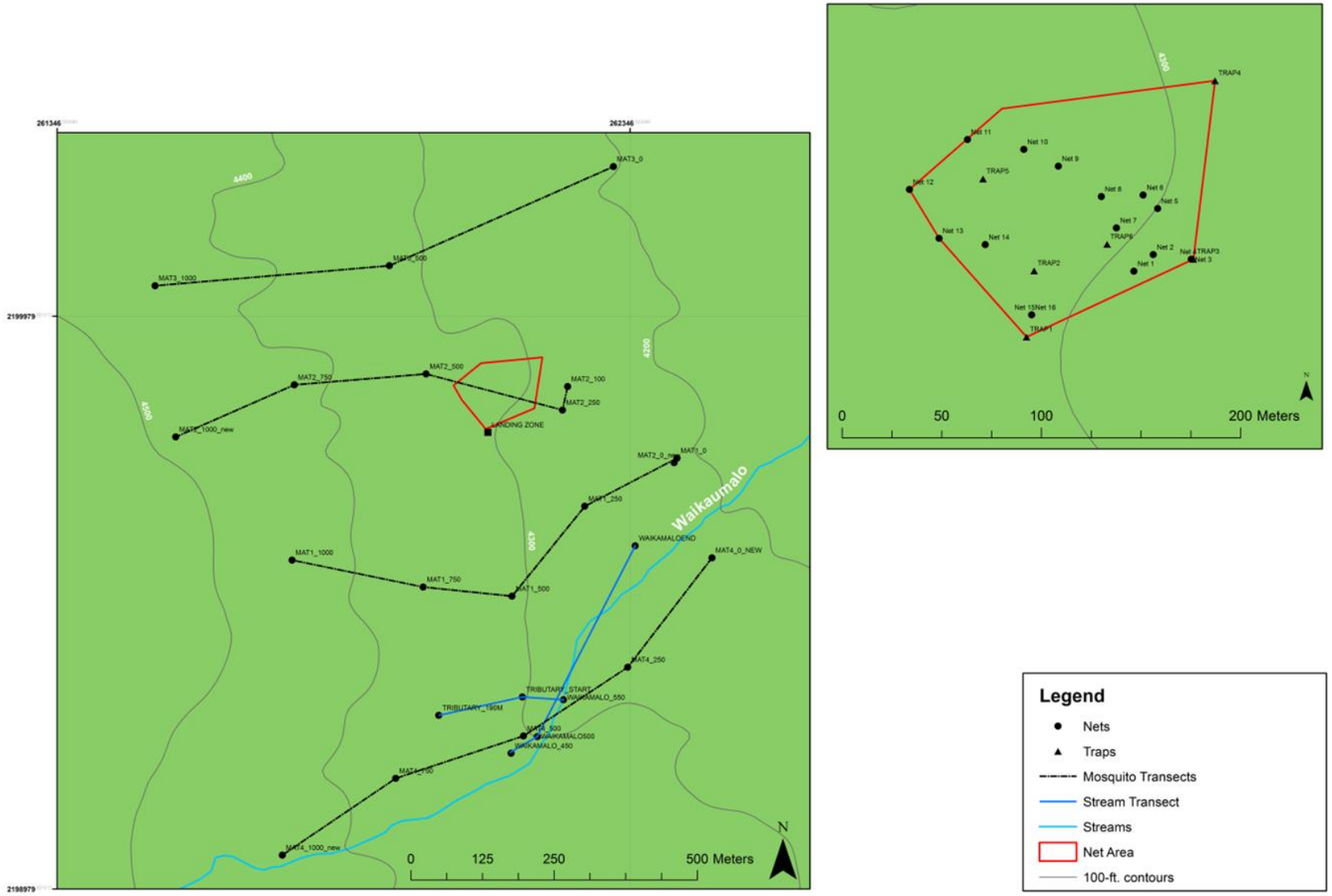
Map of the Nāuhi study site showing terrestrial and stream transects and (insert) location of mosquito traps.



Map of the upper Pua `Ākala study site showing terrestrial and stream transects and (insert) location of mosquito traps.



Map of the lower Pua`Ākala study site showing terrestrial and stream transects and (insert) location of mist nets and mosquito traps.



Map of the Maulua study site showing terrestrial and stream transects and (insert) location of mist nets and mosquito traps.

APPENDIX II. STANDARD OPERATING PROCEDURES (SOPS)

Standard Operating Procedures for Feral Pig Activity and Available Habitat Survey
ID: Culex SOP 4 Version 1

Effective Date: May 1, 1999

Scope: This SOP concerns standard belt transects conducted to quantify both feral pig activity and available habitat for larval mosquitoes. The feral pig activity survey is based on methods developed by land managers in Hawai`i (Anderson and Stone, 1993) and utilizes a standard set of pig sign and criteria for subjective age grading. Each kilometer long transect is five meters wide (2.5 meters to either side of middle) and divided into ten meter sections which are evaluated separately. This data is intended to provide an index of pig density, intensity of damage, type of damage and relative age of damage. The available habitat component of the survey is intended to quantify and identify type of aquatic habitat available to larval mosquitoes. Hapu'u and general habitat type are also recorded as they are relevant factors when comparing transacts and sites.

Procedures:

1. **Selecting transect sites.** Initial selection of transect sites is to be made from topographical maps and modified as necessary by field constraints. Transects are to be located off forestry roads, trails or bird transacts. Forest transects will follow slopes at an angle which will encounter most topographical features (ridges, bogs, intermittent streams, etc.) or transverse the width of narrow plateaus. Stream transects will follow a continuous shallow-banked section of stream. Three to four parallel transects will be spaced 500 or 250 meters apart to cover an approximate one square kilometer. Short adjacent sections of forest transects will be spaced 50 meters apart.
2. **Labeling and flagging of transects.** Transects labels (code) consist of a three letter site code followed by a transect number and section letter when applicable (Short adjacent sections of transacts are identified as A, B, C..... Z.). An example might be RRT-1 A for Reynolds Ridge Transect – Transect 1, Section A. The origin point of a transect (or transect section) will be marked by a maypole of orange, blue and pink flagging. A live woody plant should be selected for the maypole. In areas frequented by the public, origin markers should be observable from the trail but not on the trail. The transect label and *0 meters* should be marked on the orange flagging. **All field labels** will **be made with permanent marker or grease pencil.** An aluminum tag bearing the embossed *USGS-BRD-PIERC Culex Project,* transect label, *0 meters,* and compass heading should be attached at eye level to nearby vegetation. The end of transects should be marked by a similar maypole and tag and labeled 1 km. When transects are accessed at mid-length a *500 m* maypole and tag will be present. Transects are flagged every ten meters with orange flagging. Orange flags will be marked with the distance in meters from the origin. In dense vegetation and on paths around obstacles blue flagging should be added to aid in navigation. Pink flagging should be used to indicate larval habitats to be sample. **All flagging should be placed securely to live woody plants at eye level.**

3. **Recording data and the data form.** All data is recorded in pencil on the Pig Activity and Water Source Data Sheet. The data sheet should be protected from water and mud as much as possible. All data should be recorded within the box provided, in clear legible printing. Completed data sheets will be dried and stored together in a file at the end of each day. *Date* - The day on which the survey was conducted. The format is month-day-year. *Weather* **-** The weather on the day survey. Circle one or more. *Transect* **-** Transect code. *Observer* **-** The initials of those individuals observing and recording, respectively*. Time* - The time starting and ending that section of transect (O -500 m maximum). Format in military time, i.e. 0730. *Meters* **-** Distance from origin, 10, 20, 30..... 500, 510, 520, 530....1OOO. Record entire number. (*Station)Coordinates* - GPSed coordinates in UTM for 0, 500, and 1000 meters. An example for coordinates at 5OO meters might be (500) 05Q O268856 UTM 2166579 ± 17. *Digging – Misc. Sign* –If sign is present score the age according to standard criteria sheet. **Note only digging may be aged very old.** A transect section may contain fresh, intermediate, old and very old sign. Record sign age as F, I, O or V. Sections without sign should be scored with a horizontal slash*. Hapu`u #* **-** Tree fern *(Cibotium and Sadleria)* of 4 inch or greater diameter. Refer to hapu`u trunk model. Live hapu'u must have green fiddleheads and/or fronds. Dead hapu'u must have **evidence of pig feeding**. Record as a whole number. *Cavities* The number of cavities in hapu'u of 4-inch diameter or greater which can hold water. Wet cavities must contain water. Dry cavities are not wet but must be able to hold water. *Wallows* **-** The number of wallows. Wet wallows contain water. Dry wallows contain no water. *Misc.* Number of miscellaneous larval habitats. Record as number and type code separated by comma. *Ground Pool (GP*) Standing water that is not ephemeral in nature. Substrate and submerged vegetation differs from adjacent dry areas. Contains aquatic invertebrates. May be in a forest or bog. *Tree Hole (TH)* Cavity in standing or fallen tree. *Rock Hole (RH)* Depression in rock surface that collects water. Common in stream beds. *Pond (PD)* Large body of standing water. Uncommon in natural areas. *Stream (ST)* Permanent swift flowing stream and the still pools along its margin. Exposed rocky substrate. *Seep (SP)* Small wet depression with associated drainage. *Intermittent streams (IS)* Small, narrow and often steep banked streams that flow only during or following heavy rains. Often fern covered with muddy substrate and little exposed rock. *Terrain* –Descriptors for general terrain within transect section. Use only one descriptor per section unless in a forest with sphagnum ground cover *(F,Sp).* Choose descriptor that pertains to > 50% of the transect section. Choose *S* if a stream or intermittent stream is present in the section*. Site #* **-** Place a check in this box if a site should be sampled later. For existing sites record the id number of sample sites located on or from the transect section. Also if the existing site is dry, record *D* in margin, if the site no longer exist (cavity has rotted out) record *R* in margin.
4. **Survey calibration.** Prior to conducting a survey at a new site all observers should undergo a calibration exercise. For the calibration exercise thirty meters of transect is laid out with line and flagging. Flagging is also placed out on either side to mark the 2.5-meter distance. All observers record data in calibration books and compare data after each section. An additional 60 meters is than recorded and compared section by section until all observers are in agreement on signs, age and hapu'u number (< 1 per 10 counted). Calibration logs will be photocopied and filed after each calibration exercise.
5. **Conducting the survey.** Each transect is conducted by two individuals. The lead individual establishes the transect and observes for pig sign. Second individual observes

hapu'u number, aquatic habitats and terrain. Second individual also records the data, flags obstacles and potential sites, and GPSs coordinates. Record date, transect weather, observers and time. Flag, tag and GPS the transect origin. Lead ties off hip chain and walks transect at pre-determined compass heading. Be careful to keep all metal, especially machetes, away from compass. Second individual should be careful not to break hip chain. Tie off broken hip chain immediately. Clearing of vegetation is only undertaken in uluhe or very dense understory. **Machetes should be sheathed when not clearing vegetation.** Every ten meters, flag and label with the distance from origin. Data for each section should be recorded before proceeding to the next ten meters. At 500 m record time out, GPS and fill out new data sheet. At 1000 m flag, tag and GSP location.

Pig activity criteria guide

Digging
A. Fresh (F)
Fluffy soil; soil clumps on rootlets; digging moist in comparison to surrounding dry soil, but dependent upon weather; litter distribution uneven or different from surrounding. Dug-up plants green, not withered or wilted.
B. Intermediate (I)
No seedlings, or seedlings with cotyledons only; litter distribution uneven, but with pockets of continuous litter layer; dug-up plants partially green with browning leaf tips.
C. Old (O)
Seedlings emerging; litter cover uniform and/or accumulated in pits; dug-up plants drying, dead, or re-rooting.
Trails and tracks
A. Fresh (F)
Green and broken vegetation; fresh scats; tracks well-defined, edges of prints sharp or not eroded; soil in print marks moist looking, different from surrounding soils.
B. Intermediate
Broken vegetation browning, trampled; track prints slightly eroded.
C. Old (O)
Untrampled look; regrowth of vegetation; eroded track pattern.
Scat
A. Fresh (F)
Odor; steaming; mucus coating; wet-looking (dependent upon weather); flies or other insect activity; fresh tracks or plant feeding nearby; does not crumble when smashed.
B. Old (O)
Gray or black, eroded; seedlings emerging; hardened; fragmented; dung beetles; fungal growth on scat.
Plant feeding
A. Fresh (F)
Damaged plant material green, fresh-looking; uprooted plants

green; soil still clinging to exposed rootlets.
B. Intermediate (I)
Affected plant material browning.
C. Old (O)
Plant material brown and dead; eaten plant shoots regenerating; regrowth of shoots from tubers, rhizomatous or corm plants; uprooted plants dead or re-rooted; vertical plant growth from horizontally lying plants.

Anderson, S. J. and C. P. Stone. 1993. Snaring to control feral pigs *Sus scrofa* in a remote Hawaiian rain forest. Biological Conservation. 63: 195-201

Standard Operating Procedure for Larval Habitat Characterization and Sampling
ID: Culex SOP6 Version 1

Effective Date: August 23, 1999

Scope: This SOP concerns the characterization and sampling of aquatic habitat available to larval mosquitoes. An accurate location of the site is made and a number of physical, chemical and biotic parameters are recorded before sampling for mosquito larval and associated invertebrates. Some of the parameters are subjective descriptors so it is important to understand the criteria defining these parameters. The primary purpose of this study is to identify the larval habitat of *Culex quinquefasciatus* and the key parameters defining it. Secondarily, this study is an inventory of non-mosquito aquatic organisms. Ultimately this information will be used develop environmentally sound larval mosquito control strategies.

Equipment, Selecting sites, Labeling and General Habitat Characterization

Procedures:

1. **Equipment** – The sampling procedure is somewhat equipment intensive so be sure to have everything you need before heading into the field. Refer to checklists for equipment needs. Make sure all meters and altimeters are properly calibrated. Altimeters should be checked against known benchmarks in the area each day. Refer to Culex SOP5 for meter calibration.
2. **Selecting Sites** – Some sites will have been flagged during the pig activity surveys. Refer to Culex SOP4. As the minimum sample number per transect is twenty sites, additional sites may need to be located off transect. Select sites by habitat type in accordance to proportions recorded during pig activity survey (i.e. If on transect you counted 5 hapu`u cavities, 3 ground pools and 2 stream margins than you should try to sample 10 hapu`u cavities, 6 ground pools and 4 stream margins.). Always sample unique sites (i.e. tree holes). When mosquitoes are present, try to sample the same habitat types with and without mosquitoes (i.e. ten hapu`u cavities sample five with mosquitoes and five without). The twenty sites minimum is not always possible in some localities. Additional sampling is always encouraged.
3. **Flagging and Labeling Sites** – Sites should be marked with a pink flag (in some cases a pink and blue flag have been used) and labeled with an aluminum tag. Attach the

aluminum tag and flagging to nearby vegetation. The transect code and site number should be embossed on the tag along with the agency/project identification (USGS-BRD-PIERC Culex Project). Pink flagging is also used to blaze a trail to off-transect sites.

4. **General Data Recording** – Upon reaching a site to be sampled turn on the GPS unit and set it down in a safe spot. Record the following data on "Larval Habitat Survey" data forms in pencil. Consult partner on subjective parameters. *Date:* Date sampled, recorded in month-day-year format. *Time:* Time sampled, recorded in military 24-hour format. *ID Number:* Transect code – Site number. *Location:* General geographical location taken from topographical map i.e. Hakalau NWR, Pua `Ākala. *Elevation:* Read from the altimeter, recorded in meters. *Coordinates:* Record from GPS unit in UTM format. Include ± average accuracy. If there is no satellite availability, then record "NA". Save coordinates to GPS unit. Refer to Culex SOP3 for operating Garmin GPS units. *Vegetation:* Dominant/subdominant canopy species; Dominant/ subdominant understory species i.e. Ōhi`a /koa; ōlapa/hapu`u. Refer to plant/plant community sheets. *% Canopy:* Subjective estimate of canopy cover directly overhead and recorded as: < 10, 10–25, 25–60 or > 60. % Understory: Subjective estimate of understory cover directly overhead and recorded as above. *Site Location: Station*: Transect section in which site is located within or sighted from (nearest orange flag). *Compass Bearing:* Heading from transect flag toward off-transect site. *Distance:* Estimated distance to site in meters.
5. **Habitat Type** – Circle the most appropriate habitat type as defined below. *Tree hole:* Rot hole in tree holding water. Most commonly observed in windfall koa*. Nat. Container:* Natural receptacle i.e. rat gnawed coconut. *Ground Pool:* Standing water that is not especially ephemeral. Substrate and vegetation differs from adjacent dry area. May be in a bog. *Roadside Ditch:* Drainage along road or depression in road. *Leaf axil:* Leaf whorls that capture water, i.e. bird-nest fern. *Art. Container:* Bottle, can, tire, styrofoam bento box etc. *Wallow:* Pig created pool usually oval shaped with smooth sides of fine mud. *Stream Margin:* Backwater and isolated pools on banks of permanent streams or pools in muddy bottom of intermittent streams. *Rock Hole:* Depression in solid rock. Common feature in dried out "permanent" streams. *Trough:* Livestock watering container. *Pond:* Permanent still water of reasonable depth and size. Usually in pastureland. *Cistern*: Containment for human water needs. Reservoir. *Hapu`u Cavity*: Hollowed out section of tree fern that collects water. May be created by: *abscission scar* rotting – clearly defined small hole shaped like a cross section of frond stem; feral *pig feeding* – large hole, damage often done to the whole fern; *rats* – teeth marks evident usually in association with other causes; or *human* activity - straight machete or chain saw cut.

Sampling the Site

Procedures:

1. **Setting up to Sample** – Upon arrival at a site turn on both meters. Place the temperature probe of pH meter such that the metal probe is in the air and not touching any surface. Position probe out of direct sunlight. Set DO meter to appropriate altitude (elevation). Record all data on "Larval Habitat Survey" data form.
2. **Taking a Bacteria Sample** – Bacteria samples are only taken from a subset of sites, eight per transect. Select sites to be sampled "randomly" but try to sample different habitat types, with and without mosquitoes. To take a sample, first remove the cap from a formalin vial. Next open the pipette wrapper and insert pipette into the pump without

touching the sterile tip. Now insert the tip 1 cm below the water surface and slowly draw up 4.5 milliliters being careful not to suck up sediment and algae. Decant entire 4.5 ml into formalin vial, recap tightly and label the vial with the date, site id number and a two letter habitat code (Culex SOP4). Check off bacteria sample box on data form. Bacteria samples should be kept cool at all times. Refrigerate samples immediately upon return from the field.

3. **Taking a Water Sample for Meters, etc. –** Collect 50 ml of water in the plastic beaker being careful not to unnecessarily agitate the water. Allow sediment to settle. Examine the water and record its color. Circle only one color. Determine turbidity by holding beaker against data sheet. If you can read through it then circle clear, if you cannot read through it then circle turbid. Next measure the dissolved oxygen with the DO meter. Record both percent and ppm. Refer to Culex SOP5. Rinse off DO meter membrane with clean water. Next record the ambient temperature from the pH meter, measure the pH of the water sample and record the water temperature. Record both pH and mv. Refer to Culex SOP5. Rinse off pH electrode.
4. **Measuring Habitat Size –** *Dimensions*: Depending on the habitat type this will require a rule or tape measure. For measuring cavities not completely full of water, first determine the point where water will overflow. Base measurements on an imaginary level plane extended through this point. Record length, width and depth in that order, i.e. 5x6x12 cm. Measurements are to be in centimeters (cm) unless another unit is written in. *Volume:* This is the actual total volume of water sampled and recorded in milliliters as read from a graduated cylinder (i.e. 3450 ml) or the total number of standard dips taken at a site (i.e. 10 dips). Refer to invertebrate sampling below.
5. **Other Parameters –** *Substrate:* Substrate of the aquatic habitat. Leaf litter means intact leaves. Starch means the exposed soft core of the hapu`u. Other substrate types should be self-explanatory. Circle as many as apply. *Hapu`u Cavity Age:* Circle one based on the Pig Activity Criteria Guide. *Permanence:* Does the habitat exist year round. Very subjective. Circle either permanent or temporary. *Algae Present:* If algae are present check off the box on the data form. Algae may be filamentous or encrusting. *Notes:* Observer may embellish on any parameter. *Initials:* First initials of the recording observer then field partner. i.e. DAL/ADL.
6. **Invertebrate Sampling** – After all the data is collected the site may be sampled for mosquitoes and other aquatic invertebrates. For small sites, under three liters, place the two nested sieves on top of the graduated cylinder. The larger mesh should be on the top. Collect water from the site with a turkey baster and pass water through the sieves. Sieves may need to be gently tapped to keep the screens clear to flow. Negative and positive pressure created by the baster may also restore flow when sieves are clogged. Be sure to add the water sample used for the meters. Record the final volume from the graduated cylinder, return water back to the site and flush out the sieves into a sample bag with clean water. Record date and site ID number on the bag. Re-sieve the water from the site, return the water to the site and flush the sieve into the sample bag. For larger sites use the mosquito dipper. Ten full dips is the standard sample size. To dip, gently angle the cup into the water being careful not to cast a shadow on the water surface. Pass the entire contents of each dip through the sieves. Take ten dips mostly from the edge and vegetation and only a few across the deeper middle. Flush out the sieves into a labeled sample bag and be sure to record the number of dips taken under *volume.* Some sites are very shallow and it is difficult to take a full dip. Adjust the number of dips to reflect ten full dips, i. e. twenty half dips. Some sites are too large for

the baster and too small for ten dip. In these situations just record the actual number of dips.

Standard Operating Procedure for Dip Surveys
ID: Culex SOP7 Version: 1

Effective Date: September 24, 1999

Scope: This SOP concerns the intense sampling of streams, drainages and open water bogs. The purpose of a dip survey is to attempt adequate sampling effort for detection of mosquito larvae where the aquatic habitat is extensive. Dip surveys are conducted along with water source and larval habitat surveys along streams or from established trails through boggy areas. They are usually 1 kilometer in length but may vary.

Procedures:

1. Since the dip survey is conducted along with water source and larval habitat surveys, a minimum team for a narrow stream survey would be two individuals. On large streams a four-member team may work best.
2. Stream transects are assigned three letter codes based on the streams name (i.e. MST = Mohihi Stream Transect). Trailside transects are assigned similar codes utilizing the name of trail. When other transects use the same code the trail transect will be numbered 0 (i.e. AST – 0 = Alakai Swamp Trail boardwalk). Refer to Culex SOP4.
3. The lead person will hip chain the distance and hang a numbered orange flag (stream survey), or verbally indicate distance (trail survey), every ten meters. This person will also record the type and number of aquatic habitats per 10-meter section and the GPS coordinates for the origin, middle and end of each transect. Refer to Culex SOP4.
4. Dips will be made by all members of a team with a standard mosquito dipper attached to a meter-long handle. To dip, gently angle the cup into the water so as to draw surface water into the cup. Do not plunge the dipper in deep. Do not cast a shadow on or disrupt water surface while dipping. Take most dips from the edge of the habitat and among submerged vegetation.
5. A minimum of ten dips per 10-meter section should be made, while attempting to sample each distinct rock hole or ground pool. If the habitat is limited in a section, then make up the number of dips in the next section.
6. For each dip observe the presence or absence of mosquito larvae, veliids, copepods (cp), ostracods (os), odonates (od), dytiscid (dy) and chironomids (ch). Refer to Little Bug Key for identifying features.
7. One observer will record all the team observations in a Stream Dip Survey log. General information: *Location*: Transect code. *Date*: month-day-year. *Observers*: Initials of team members starting with the recorder of the Stream Dip Survey log. The log is set up for 250 meters per page. Distance is to be recorded in the *Location* column and *Time* need only be recorded at the beginning and end of each 250-meter section. Number of dips, dips with *Culex* and dips with veliids will be recorded by hash marks at ten marks per line. Other invertebrates observed should be recorded as their two-letter code (above) followed by the number of dips they were observed in. Lists of invertebrates should be delimited by commas (i.e. cp 6, os 7, od 1).

8. Collect a few representative specimens along the transect. Preserve these in ethanol and label with date and location as recorded in log.
9. While conducting the survey select, at "random", twenty sites to be fully sampled on the way back. Flag these sites with pink but do not dip until conducting the complete larval habitat survey. Refer to Culex SOP6.

APPENDIX III. AGE DISTRIBUTION OF NATIVE BIRDS SAMPLED AT HAKALAU FOREST NATIONAL WILDLIFE REFUGE.

Year	Site	Species	Total	HY	AHY	SY	ASY	TY	ATY
1998	Nāuhi	AKEP	7	0	0	4	0	0	3
		AKIP	4	1	1	2	0	0	0
		APAP	50	4	0	17	29	0	0
		HAAM	92	17	0	15	60	0	0
		HAEL	66	0	0	16	0	12	38
		HCRE	19	3	15	0	1	0	0
		IIWI	118	6	1	12	99	0	0
		OMAO	27	2	24	1	0	0	0
	Site Total		383	33	41	67	189	12	41
	Maulua	APAP	16	1	0	3	12	0	0
		HAAM	24	2	21	0	1	0	0
		HAEL	6	0	2	0	4	0	0
		IIWI	13	3	8	0	2	0	0
		OMAO	9	1	5	3	0	0	0
	Site Total		68	7	36	6	19	0	0
	Pua `Ākala	APAP	12	0	0	2	10	0	0
		HAAM	6	0	6	0	0	0	0
		HAEL	13	0	0	1	3	3	6
		IIWI	21	3	17	0	1	0	0
		OMAO	9	0	9	0	0	0	0
	Site Total		61	3	32	3	14	3	6
2012	Nāuhi	AKEP	1	0	0	0	0	0	1
		APAP	30	1	1	5	23	0	0
		HAAM	76	0	16	34	26	0	0
		HAEL	19	0	0	0	6	0	13
		HCRE	3	0	3	0	0	0	0
		IIWI	89	6	3	24	56	0	0
		OMAO	23	0	1	12	10	0	0
	Site Total		241	7	24	75	121	0	14
	Maulua	APAP	5	1	0	2	1	0	1
		HAAM	4	0	1	2	1	0	0
		HAEL	5	1	0	2	0	2	0
		IIWI	12	3	7	2	0	0	0
		OMAO	1	0	0	1	0	0	0
	Site Total		27	5	8	9	2	2	1
	Pua `Ākala	HAAM	6	0	0	2	4	0	0
		HAEL	12	4	0	2	0	2	4
		HCRE	1	1	0	0	0	0	0
		IIWI	1	0	1	0	0	0	0
		OMAO	3	0	1	1	1	0	0
	Site Total		23	5	2	5	5	2	4
	TOTAL		803	60	143	165	350	19	66

HY = Hatch Year, AHY = After Hatch Year, SY = Second Year, ASY = After Second Year, TY = Third Year, and ATY = After Third Year.

APPENDIX IV. PREVALENCE OF AVIAN MALARIA (*PLASMODIUM RELICTUM*) AT HAKALAU FOREST NATIONAL WILDLIFE REFUGE.

Species	Percent Malaria Prevalence (Infected/Total)							
	Nāuhi 1600 m		Pua `Ākala 1300 m		Maulua 1300 m		Low Elevation Totals	
	1998	2012	1998	2012	1998	2012	1998	2012
`Ōma`o	13 (3/24)	13 (3/24)	57 (4/7)	0 (0/3)	11 (1/9)	100 (1/1)	31 (5/16)	25 (1/4)
Hawai`i `elepaio	7 (3/45)	0 (0/19)	0 (0/11)	0 (0/12)	50 (3/6)	0 (0/5)	18 (3/17)	0 (0/17)
Hawai`i `amakihi	12 (9/77)	3 (2/79)	14 (1/7)	0 (0/6)	25 (6/24)	25 (1/4)	23 (7/31)	10 (1/10)
Resident Subtotal	10 (15/146)	4 (5/122)	20 (5/25)	0 (0/21)	26 (10/39)	20 (2/10)	24 (15/63)	7 (2/31)
`Apapane	20 (9/45)	10 (3/30)	42 (5/12)		25 (4/16)	0 (0/5)	32 (9/28)	0 (0/5)
`I`iwi	9 (10/107)	1 (1/89)	10 (2/20)	0 (0/1)	18 (2/11)	0 (0/12)	13 (4/31)	0 (0/13)
Vagile Subtotal	13 (19/152)	3 (4/119)	22 (7/32)	0 (0/1)	22 (6/27)	0 (0/17)	22 (13/59)	0 (0/18)
Hawai`i `Ākepa	0 (0/6)	0 (0/1)						
`Akiapōlāa`u	0 (0/4)							
Hawai`i creeper	0 (0/13)	0 (0/3)		0 (0/1)				
Endangered Subtotal	0 (0/23)	0 (0/4)		0 (0/1)			0 (0/0)	0 (0/1)
Native Subtotal	11 (34/321)	4 (9/245)	21 (12/57)	0 (0/23)	24 (16/66)	7 (2/27)	22 (28/123)	4 (2/50)
Japanese bush warbler						0 (0/1)	0 (0/0)	0 (0/1)
Japanese white-eye	17 (3/18)	19 (4/21)	7 (2/28)	0 (0/3)	11 (2/19)	38 (3/8)	8 (4/47)	27 (3/11)
Northern cardinal	0 (0/2)	0 (0/2)						
Red-billed leiothrix	8 (4/50)	0 (0/14)	0 (0/13)	0 (0/7)	9 (1/11)	0 (0/17)	4 (1/24)	0 (0/24)
Yellow-fronted canary		0 (0/1)						
House finch	100 (1/1)							
Melodious laughingthrush			(0/1)				0 (0/1)	100 (1/1)
Non-native Subtotal	11 (8/71)	11 (4/38)	5 (2/42)	0 (0/10)	10 (3/30)	12 (3/26)	7 (5/72)	11 (4/37)
Community Total	11 (42/392)	5 (13/283)	14 (14/99)	0 (0/33)	20 (19/96)	9 (5/53)	17 (33/195)	6 (5/87)