

## INCREASE IN AVIAN MALARIA AT UPPER ELEVATION IN HAWAII

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**Abstract.** Hawaiian honeycreepers (Aves: Drepanidinae) evolved in the absence of mosquito-borne diseases such as avian malaria (*Plasmodium* spp.). Malaria has been largely responsible for the recent wave of extinctions and endangerment among Hawaiian forest birds, which began by the 1920s. Most honeycreepers, especially endangered species, now persist only in forests above 1500 m elevation, where cool temperatures prevent effective malaria development in mosquitoes. We report here that prevalence of malaria in Hawaiian forest birds at 1900 m on the island of Hawai'i has more than doubled over a decade. This increase was associated with breeding of mosquitoes and warmer summertime air temperatures. We also report direct evidence of tolerance to malaria, and a possible cost of tolerance, in wild native birds. Tolerance is adding to a reservoir of malaria at upper elevations even while vectors are rare and air temperatures are too low for complete development of the parasite in the vector. The data provide a glimpse of how malaria is becoming an emergent infectious disease at upper elevations.

**Key words:** avian malaria, climate warming, cost of tolerance, Hawaiian honeycreepers, mosquito vector, tolerance.

### Aumento en la Prevalencia de Malaria Aviar en las Tierras Altas de Hawai'i

**Resumen.** Los Drepanidinae de Hawaii evolucionaron en una ambiente carente de enfermedades transmitidas por mosquitos, tales como la malaria aviar (*Plasmodium* spp.). La malaria ha sido responsable en gran medida de la ola reciente de extinciones y de la amenaza que pesa sobre las aves de bosque de Hawaii, un proceso que comenzó en la década de 1920. La mayoría de las especies de Drepanidinae, especialmente las amenazadas, sólo persisten actualmente en bosques localizados por encima de los 1500 m de elevación, donde las temperaturas frescas previenen el desarrollo efectivo de la malaria en los mosquitos. En este estudio documentamos que la prevalencia de malaria ha aumentado a más del doble a lo largo de una década en las aves de bosque a 1900 m en la isla de Hawai'i. Este incremento estuvo asociado con la reproducción de mosquitos y con temperaturas del aire más cálidas en los veranos. También presentamos evidencia directa de tolerancia a la malaria, y de un posible costo como consecuencia de dicha tolerancia, en aves silvestres nativas. La tolerancia está creando un reservorio de malaria a mayores elevaciones, aún cuando los vectores son escasos y las temperaturas del aire son demasiado bajas para que el desarrollo del parásito se complete dentro del vector. Estos datos proveen una indicación de cómo la malaria está convirtiéndose en una enfermedad infecciosa emergente en las tierras altas.

### INTRODUCTION

Hawaiian passerine forest birds, especially the Hawaiian honeycreepers (Drepanidinae), are renowned among evolutionary biologists for extensive adaptive radiation of morphology, plumage, and behavior (Amadon 1950, Freed et al. 1987). There were 53 historically known endemic passerine species and subspecies from the

main islands (Scott et al. 2001), as well as numerous taxa known only from the subfossil record (James and Olson 1991). These same birds provide conservation biologists with a premier example of extensive extinction and endangerment (Freed 1999). Forty percent of the historically known taxa are extinct, and more than one half of those surviving are federally listed as endangered (Scott et al. 2001). The most recent extinction occurred in 2004 (Downes 2004). These birds have been subjected to direct exploitation for their feathers (used in human adornment, Kirch 2000), wholesale destruction

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of habitat for human agriculture and cities, degradation of habitat through cattle ranching and introduction of pigs for hunting (Cuddihy and Stone 1990), and biological interactions with introduced species. These interactions include predation, competition, parasitism, and infectious disease (van Riper and Scott 2001).

Introduced infectious diseases such as mosquito-borne avian malaria (*Plasmodium relictum*) and avian poxvirus (*Poxvirus avium*) are especially important because of evolutionary considerations (Ewald 1994, Combes 2001). Based on mitochondrial DNA comparisons, the common ancestor of Hawaiian honeycreepers would be approximately 5 million years old (Fleischer et al. 1998). The exposure of the birds to these diseases is, by contrast, quite recent. No known vector of malaria or poxvirus for forest birds has been identified in the native insect fauna (Nishida 1997). The major mosquito vector (*Culex quinquefasciatus*) of malaria was unintentionally introduced to the coast of Maui in 1827 (Hardy 1960). Numerous birds from different continents were introduced in the late 19th and early 20th century (Moulton and Pimm 1983). The malarial pathogen (*P. r. capistranoae*) was likely present in some of these, especially the Blue-breasted Quail (*Coturnix chinensis*) from the Philippines, the type host for the *Plasmodium* identified in Hawaiian birds (Laird and van Riper 1981, Laird 1998). Two other mosquito species, *Aedes albopictus* and *Wyeomyia mitchelli*, which can transmit malaria (LaPointe 2000), were introduced later. Given the importance of infectious diseases in birds that have coevolved with them (Moeller 1997, Valkiunas 2005), the evolutionary mismatch between Hawaiian birds and the introduced vector and pathogens is apparent, as may be the case with other examples of emerging infectious diseases (Daszak et al. 2000, Dobson and Foufopoulos 2001). The immune system of the honeycreepers has not been shaped by the selective pressure of avian malaria or poxvirus for five million years.

By the time the first studies of malaria were conducted in the 1960s and 1970s (Warner 1968, van Riper et al. 1986), one third of all native forest bird species were extinct, and surviving species had contracted in range and abundance (Warner 1968, Pratt 1994). Distributional anomalies, defined as absence or rarity of birds in what appeared to be suitable forest, were de-

tected in the comprehensive Hawai'i Forest Bird Survey conducted in the 1970s (Scott et al. 1986). Native birds, especially endangered birds, were not found in suitable forest at mid or low elevations, where alien bird species were most abundant but did not appear to be competing with the natives (Mountainspring and Scott 1985, Scott et al. 1986). An elevational limit of malaria was documented at approximately 1500 m during the 1970s (van Riper et al. 1986). The mosquito vector becomes rare above this elevation (Goff and van Riper 1980), and effective transmission of malaria is further reduced because low temperatures (<13°C) limit the development of sporozoites (= sporogony), the stage of malaria infectious to birds (LaPointe 2000).

The situation in Hawai'i is widely used as an example of how an introduced pathogen can decimate a fauna (Dobson and May 1991, McCallum and Dobson 1995). Most endangered species and other species highly susceptible to malaria currently exist only between 1500 m and the upper limit of their forest habitat around 1900 m (Scott et al. 1986). Climate warming could raise the 'mosquito line', modeled on the sporogony threshold of 13°C, from 1500 m to nearly 1900 m within the next 100 years (Benning et al. 2002, Harvell et al. 2002). Such warming would allow mosquitoes and local cycles of malaria transmission to reach almost all remaining forest bird populations in Hawai'i. At issue is whether tolerance or resistance to malaria can evolve rapidly enough for susceptible species to avoid extinction (Freed 1999). The last Po'o-uli (*Melamprosops phaeosoma*), which died in captivity, was infected with avian malaria (E. VanderWerf, pers. comm.).

Tolerance and resistance are related to stages of the malarial infection, and infections may be acute or chronic. After a bite by an infectious mosquito, plasmodia may show up by microscopy of stained blood smears within a week (van Riper et al. 1986). The acute phase occurs 12–28 days after the bite, and is associated with the highest level of parasitemia (parasites per red blood cell, van Riper et al. 1986). During the acute phase, symptoms of caged Hawaiian birds experimentally infected with malaria included opening and closing the eyes, fluffing of plumage, and reduced food consumption (Atkinson et al. 1995, 2000). Hayworth et al. (1987) showed that infected birds lower their body temperature

during this phase. High mortality of experimentally infected Hawaiian birds occurred during the acute phase (Atkinson et al. 1995, 2000). The mortality and morbidity during this phase are the primary reasons that sick birds are rarely captured in mist nets (Valkiunas 2005). The chronic phase follows, with a drop in the level of parasitemia that can extend for up to 120 days before becoming no longer detectable by microscopy (Atkinson et al. 2000). Resistant birds are those that clear the infection before the acute phase occurs. Tolerant birds are those that survive the acute phase. Studies of experimentally infected birds reveal that tolerant birds likely retain the chronic level of infection for life (Atkinson et al. 2001, Valkiunas 2005). Weakening of the immune system by stress or an excessive increase in energy expenditure can result in recrudescence of the chronic infection into higher parasitemia levels that are again detectable by microscopy (Norris et al. 1994).

Some Hawaiian forest birds are evolving in response to malaria. Tolerant birds, more strictly defined as infected individuals that survive and breed successfully, have not been reported at upper elevations, but are known from lower elevations in common species such as the Hawai'i 'Amakihi *Hemignathus virens virens*, Woodworth et al. 2005). Resistant birds, defined as individuals in which the pathogen does not get established after transmission, may be present in an O'ahu 'Amakihi (*Hemignathus flavus*) population at low elevation (Shehata et al. 2001). Hawaiian birds at upper elevations provide an exceptional opportunity to document the evolution of tolerance and resistance to malaria as an emerging infectious disease.

Our study group was the first to document malaria, 12–16 years ago, in native birds at the upper limit of the range of endangered birds inhabiting native 'Ōhi'a lehua (*Metrosideros polymorpha*)–Koa (*Acacia koa*) forest (Feldman et al. 1995). The site was at 1920 m elevation on the island of Hawai'i. From 1988–early 1992 (= early period), we used a Polymerase Chain Reaction (PCR) diagnostic targeting the small ribosomal subunit gene and detected 2.1% prevalence of malaria ( $n = 532$ ), including infections in three species of native birds (Feldman et al. 1995). Because two of the species are known to be year-round residents, some transmission likely occurred on site. We argued that the trans-

mission was probably from infectious mosquitoes carried up slope in strong winds.

Here we document a significant increase in prevalence of avian malaria at the same high elevation site during 2001–2002. The increase was associated with warmer temperatures than during the early period, and with mosquito breeding on site. It is an early warning of what might be expected from climate warming.

## METHODS

Our study was conducted at 1920 m elevation on the windward slope of Mauna Kea, on the island of Hawai'i, at Hakalau Forest National Wildlife Refuge. The site is an old growth forest of primarily 'Ōhi'a lehua trees, with a few Koa trees emerging from the canopy (Freed 2001). Three species of endangered honeycreepers are found at their highest densities here (Scott et al. 1986). They all become rarer with decreasing elevation and their ranges terminate in continuous forest at approximately 1500 m. No 'Ōhi'a lehua–Koa forest exists at higher elevations, where such forest was converted into pasture. The birds also decrease in density laterally from north to south in the refuge, in what has been identified as a distributional anomaly (Scott et al. 1986).

Birds were captured in aerial mist nets and approximately 50  $\mu\text{L}$  of blood was obtained from the brachial vein through a puncture from a sterile 26-gauge needle. Styptic powder was applied to the wound before the bird was released. Captured birds were marked with an aluminum band, and several were additionally color-banded for determination of individual survival and reproductive success. Birds were also inspected for poxvirus-like sores or swellings. Plumage condition was inspected at each capture and breakage of feathers was noted. Blood was taken from 465 birds during 2001–August 2002 (= late period), roughly comparable to the 532 birds from the same site analyzed during the early period (Feldman et al. 1995).

Blood samples were collected in 50  $\mu\text{L}$  microhematocrit tubes and immediately transferred to an Eppendorf tube containing EDTA buffer to inhibit nucleases. The tubes were stored in the shade during the day and placed in a  $-20^{\circ}\text{C}$  freezer that night. Preparation of the samples for transfer to the laboratory on the island of O'ahu involved thawing, and then adding 4  $\mu\text{L}$  of Proteinase K and 20  $\mu\text{L}$  of SDS to each tube. This

treatment dissolves membranes, degrades polypeptides, and further inactivates nucleases. In addition, this treatment makes the blood samples safe to carry aboard airplanes. At the laboratory, the samples were transferred to a  $-80^{\circ}\text{C}$  freezer. As in the earlier study (Feldman et al. 1995), total genomic DNA was obtained from the thawed samples using chloroform/phenol extraction followed by dialysis (Quinn and White 1987). This protocol provides large amounts of highly purified total genomic DNA (Freed and Cann 2003).

The samples collected for this study were analyzed with the same PCR diagnostic used for the earlier samples. While most other avian malaria primers target unique mitochondrial sequences just on the pathogen (Richard et al. 2002, Fallon et al. 2003, Waldenstrom et al. 2004), our primers target the 18s rRNA nuclear gene in both the bird host and the malarial parasite as an indicator of infection status (Feldman et al. 1995). In contrast to birds, which have approximately 380 base pairs in the portion of the 18s rRNA gene spanned by the primers, *Plasmodium* spp. have an insertion of several hundred base pairs in their portion of the 18s rRNA gene spanned by the same primers (Cann et al. 1996). Human malaria PCR diagnostics have successfully exploited this insertion and nearby portions of the gene to accurately identify malaria species, and their proportions, in mixed infections (Li et al. 1995). More of the gene has been used to establish phylogenetic relationships among malarial parasites (Qari et al. 1996). The Feldman et al. (1995) primers span two highly variable regions of the insertion separated by a highly conserved region (regions V7 and V8 in Li et al. 1995). Although not currently exploited, this characteristic of the insertion has the potential to identify strains of *P. relictum*.

Amplification products from the above procedure are scored by agarose gel electrophoresis for infection status. With suitable positive and negative controls, the same used for all samples during both time periods, a single band would represent the bird fragment and a second band, higher in the electrophoretic lane, would represent the pathogen. This protocol and that of Waldenstrom et al. (2004) are the only two for birds that combined high sensitivity with perfect accuracy on the subset of samples identified as positive by microscopy (Freed and Cann 2003).

The main difference in the diagnostic assay used here and in the earlier study is in the quality control (consistency between manufacturer's lots) for reagents available to perform PCR. For some recent samples, amplification was done using the same thermocycler used for the earlier samples. For most recent samples, we performed PCR assays in a Perkin-Elmer 2400 thermocycler, using the Feldman et al. (1995) primers with FastStart Taq DNA Polymerase (Roche Applied Science, Indianapolis, IN) and the manufacturer's reaction buffer according to the following protocol:  $95^{\circ}\text{C}$  for 6 min, followed by 35 cycles of  $94^{\circ}\text{C}$  for 40 sec,  $47^{\circ}\text{C}$  for 2 min,  $72^{\circ}\text{C}$  for 1 min, with a final extension of 10 min at  $72^{\circ}\text{C}$ . Reactions normally contained  $\text{Mg}^{+2}$  ions at  $>4$  mM concentrations. The FastStart Taq DNA Polymerase is designed to control false priming and to specifically amplify targets that exist in low copy number (Hermann and Foerzler 2002), eliminating steps often taken with the early polymerases used, such as addition of single strand binding proteins or wax beads as temporary barriers to keep the polymerase separated from the template and primers until appropriate cycling temperatures were achieved.

Because PCR diagnostics cannot distinguish acute and chronic infections, we treated infection status of individuals as a binary variable. Logistic regression was used to test the differences in prevalence using a model that included species, time period, and the interaction. The time effect was tested after the species effect since species were not sampled equally during the two time periods and differences in susceptibility among species could account for any observed change in prevalence. Analysis of deviance was used to test for effects. The time effect directly tests the hypothesis that prevalence has changed between the two time periods.

Beginning in October 2001, and continuing through June 2004, 12 oviposition sites were provided for mosquitoes. This started when we discovered a dead 'I'iwi (*Vestiaria coccinea*) on the ground with massive poxvirus sores on its ankles. The mosquito oviposition sites consisted of 5-gallon buckets filled with two gallons of an infusion of water, dead grass (including alfalfa pellets), and brewer's yeast. They were inspected for mosquito egg rafts and larvae every four days during 12-day duty cycles. A total of 142 inspections were made.

TABLE 1. Analysis of deviance for logistic regression of infection status of malaria in individual birds with effects entered into the model in the order listed. The nine species involved were tested during both the early time period (1998–early 1992) and the late time period (2001–August 2002).

Effect	df	Deviance	Residual df	Residual deviance	Pr(chi-square)
NULL	17	74.1			
Species	8	25.4	9	48.7	$P = 0.001$
Time	1	23.4	8	25.3	$P < 0.001$
Species $\times$ Time	8	25.3	0	0.0	$P = 0.001$

Weather data were obtained from a station located 3 km north of the study area at 1950 m elevation. The station was located in an open pasture (once forest) that was little changed over the 14-year interval of the study. Temperature data were collected to the nearest 0.4°C and wind speed was measured as  $m\ sec^{-1}$ , both at 15-min intervals. Data were available from 1989–1991 and 2001. No autocorrelation at any order was detected for any sets of data used for analyses, so simple parametric tests were used. Two-sided tests of climatic data were performed.

Population trend analyses from the early period through the late period were performed for species that showed significant increases in prevalence of malaria. These were used to test the hypothesis that the change in prevalence was due to an increase in host tolerance, which predicts a positive population trend. Data were number of individuals captured per mist-net hour per year. The regression analyses were based on Dennis et al. (1991). The regression is forced through the origin, the independent vari-

able is the square root of the time period between consecutive data points, and the dependent variable is the logarithm of the ratios of the consecutive standardized captures ( $N_{t+a}$  and  $N_t$ ), divided by the square root of the time period ( $a$  minus  $t$ ).

RESULTS

Prevalence of malaria during the late period was 5.4%, more than double the prevalence of 2.1% estimated during the early period. Logistic regression, using nine species with samples during each time period, indicated that this difference is highly significant, even after adjusting for differences among species (Table 1). The significant species by time interaction (Table 1) is based largely on the observation that species with the greatest increase in prevalence were those that did not test positive during the early time period, despite adequate sampling (Fig. 1). Two such species, the Hawai'i 'Elepaio (*Chasiempis sandwichensis sandwichensis*, an endemic monarchine flycatcher) and 'I'iwi, had significant increases (test of proportions,  $P < 0.01, 0.001$ , respectively). No other increases or decreases were significant ( $P > 0.05$ ). Two species of introduced birds, the Japanese White-eye (*Zosterops japonicus*) and Red-billed Leiothrix (*Leiothrix lutea*), and one native bird, the endangered Hawai'i Creeper (*Oreomystis mana*), did not test positive during either time period ( $n = 44, 32; 6, 10; 13, 23$ , respectively).

Both mobile and sedentary host species, and juvenile and adult birds were infected during the late period. 'I'iwi and 'Apapane (*Himatione sanguinea*) are nectarivorous species that generally leave the study area for lower elevations during the postbreeding summer months when there is little flowering on 'Ōhi'a lehua trees (WAK, unpubl. data). Both juvenile and adult birds were infected (4 juveniles of 8 total infected 'I'iwi, 2 of 9 'Apapane). Therefore, low-

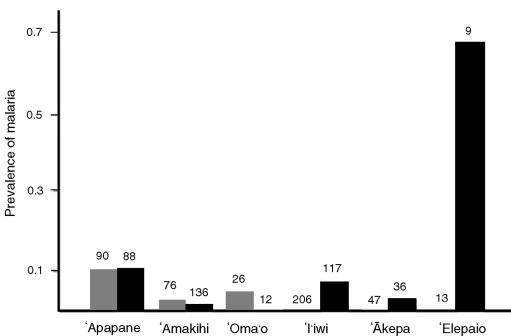


FIGURE 1. Prevalence of malaria during 1988–early 1992 (grey bars) and 2001–2002 (black bars) in six species of native birds in which malaria was detected. Numbers over bars indicate sample size for each species for each period. Numbers over the horizontal axis indicate sample sizes for zero prevalence. The positive 'Ākepa is based on symptoms.

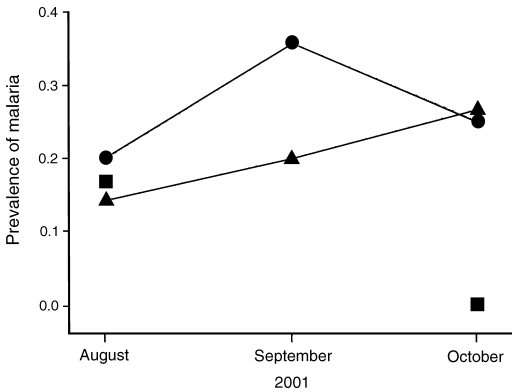


FIGURE 2. Prevalence of malaria during epizootic of 2001 for 'I'iwi (solid circles), 'Apapane (solid triangles), and Hawai'i 'Amakihi (solid squares). No 'Amakihi were captured during September. Sample sizes are in chronological order: 'I'iwi, 10, 14, 4; 'Apapane, 14, 12, 15; 'Amakihi, 6, 0, 4.

er-elevation transmission in these mobile species may account for their malarial cases at high elevation. However, the Hawai'i 'Amakihi, Hawai'i 'Elepaio, and endangered Hawai'i 'Ākepa (*Loxops coccineus coccineus*) are year-round residents, with high philopatry (Lepson and Freed 1995, VanderWerf 1998). Based particularly on prevalence in the 'Elepaio, greater on-site transmission occurred during the late period than in the early period.

The one sick endangered Hawai'i 'Ākepa was an adult male captured in November 2001 with an active poxvirus-like infection on a toe. This bird was shivering powerfully, opening and closing its eyes, fluffing its plumage, and was unable to consistently maintain its head in normal posture. This 'Ākepa was the first observed case of extended shivering in any bird we captured before 2001, in 14.5 years and over 9000 captures of all species, including 587 individuals of all species with active poxvirus infections. No blood sample was obtained from the 'Ākepa because peripheral circulation was reduced. However, the symptoms of morbidity suggest malaria. A juvenile male 'I'iwi, malaria-positive in 2001, was shivering when recaptured in March 2003. The results of the logistic regression are little changed if the 'Ākepa is not included.

The late period included a major epizootic in 2001 (Fig. 2). From January–July 2001 there was just one malaria positive bird (a Hawai'i 'Elepaio in June). During August–October 2001, 20% of 89 birds tested positive, including 'I'iwi,

'Apapane, and Hawai'i 'Amakihi. A blood sample collected 3 weeks earlier in the month from the 'I'iwi found dead on the ground in September tested positive for malaria. The sick male Hawai'i 'Ākepa detected in early November 2001 was likely part of the epizootic. There were no malaria positive birds from November 2001 through mid-August 2002 except for five Hawai'i 'Elepaio (all in 2002). Because no Hawai'i 'Elepaio were captured during the epizootic months of August–November 2001, it is not known when the 2002 birds became infected. However, four of the six positive birds were adults recaptured in mist nets up to 0.5 km apart, a distance of at least five territories.

On 1 December 2001, second instar or later mosquito larvae of *C. quinquefasciatus*, based on siphon characteristics, were detected in one oviposition bucket on the study site. No *Aedes* or *Wyeomyia* mosquitoes, mainly low elevation species, were detected in the buckets or as biting adults during the day. No mosquitoes were detected during 2002–June 2004. The observed association between mosquito breeding and the epizootic is not due to chance because, while mosquitoes were rare (one of 142 inspections), they were detected within six inspections relative to the epizootic ( $P = 0.04$ ), and within three weeks of the sick Hawai'i 'Ākepa. Our finding of mosquito breeding associated with the epizootic provides further evidence that some transmission was occurring on the study site.

To assess the influence of temperature on the epizootic, we selected those months with average temperatures above the 13°C sporogony threshold in one or both time periods (Fig. 3, July and August). Mean July–August air temperatures were not significantly higher in 2001 than in 1989–1991 ( $t_{256} = 1.6$ ,  $P = 0.12$ ). However, there were differences at a finer scale that are relevant to epidemiology. We examined runs of high temperature days that could allow outbreaks of mosquito and parasite development. The year 2001 did not have more days over 13°C, or a longer consecutive sequence of days over 13°C than did 1989–1991 (Table 2). However, for days that were above the 13°C threshold, mean air temperature was significantly higher in 2001 than in 1989–1991 (Table 2,  $t_{134} = 3.5$ ,  $P < 0.01$ ). Air temperatures during the longest sequence of days above the threshold were also significantly higher in 2001 than in earlier years (Table 2,  $t_{40} = 3.8$ ,  $P < 0.001$ ).

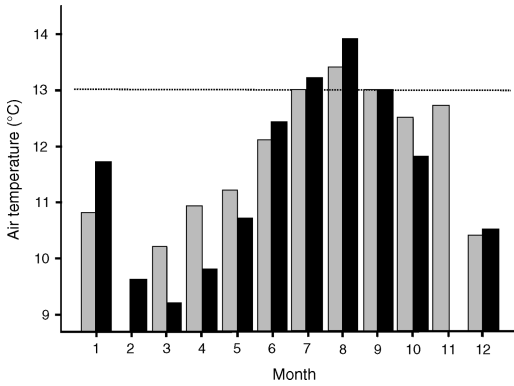


FIGURE 3. Average air temperature each month for years 1989–1991 (grey bars) and 2001 (black bars). Missing bars were due to weather station problems. Bar for September 2001 is based on only two days of data. Dotted line represents minimum temperature threshold for sporogony of *Plasmodium relictum* within the mosquito vector.

Thus, hot spells of typical duration but higher amplitude may have contributed to the 2001 epizootic. Mean wind speed did not differ among years (Table 2), and all years had a positive correlation between air temperature and wind speed for July and August. Daytime wind directions were generally NE to SE (upslope). This correlation suggests that epizootics at lower elevation contribute more to epizootics at higher elevation than would be expected by wind or temperature variation alone. Mosquitoes would be more likely to be carried upslope during the most favorable thermal conditions for the parasite.

Tolerance to disease was discovered from the epizootic. One ‘I‘iwi, an 8-year-old female who was malarial in 2001, was resighted while breeding successfully the next two years. A juvenile male ‘I‘iwi, who was malarial in 2001 at 5–10 months of age, fledged young successfully the next two years. The shivering Hawai‘i ‘Ākepa was last resighted in 2005, without nesting suc-

cessful from 2002–2005. Less is known about breeding success of the ‘Elepaio, but three of the six birds from 2001–2002 were still alive in 2005, the number expected based on their annual adult survival of  $0.83 \pm 0.06$  (VanderWerf 1998). These cases all indicate extended survival, and two indicate breeding success by both sexes. Breeding success formally documents tolerance to malaria in the wild. The ‘I‘iwi is of great interest because challenge experiments have shown this bird to be highly susceptible to malaria (Atkinson et al. 1995).

A possible cost of tolerance to malaria was also revealed by the two ‘I‘iwi. Broken head feathers have been detected in ‘I‘iwi and ‘Apapane since 2001. Both the juvenile male and the adult female ‘I‘iwi had broken head feathers when recaptured after the 2002 molt season, within a year of detection of malaria. None of the malaria-negative birds from August–October 2001 (seven ‘I‘iwi and one ‘Apapane) that were recaptured in 2002 showed this pattern. The association of feather problems and malaria among the seven malaria-tested birds cannot be attributed to chance (randomization test: 21 ways of choosing two individuals with feather problems out of seven, only one of which involves the two malarial birds with feather problems,  $P < 0.05$ ).

The trend analyses for the ‘I‘iwi and Hawai‘i ‘Elepaio had regression parameters of  $-0.39$  and  $-0.18$ , respectively. Neither parameter was significantly different from zero ( $P = 0.72, 0.31$ , respectively). Moreover, the tendency was for overall decline rather than increase.

DISCUSSION

The increase in prevalence of malaria at high elevation forests in Hawai‘i is the first documented increase for any haemosporidian parasite of birds at high elevation, and is associated with increased temperatures. The prevalence during

TABLE 2. Air temperature and wind speed during summer months. 13°C is the threshold for sporogony of *Plasmodium* in the mosquito vector.

Parameter	Year (July–August)			
	1989	1990	1991	2001
Days >13°C	28	36	37	35
Mean air temp. (°C)	14.1	14.1	14.3	14.9
Longest sequence of days >13°C	5	8	15	14
Mean air temp. (°C)	14.2	14.3	14.0	15.4
Mean wind speed (m per sec)	2.7	2.6	2.6	2.5

the epizootic was more than twice as high as earlier reports from elevations in Hawai'i as low as 1200 m (Cann et al. 1996, Woodworth et al. 2001), and was higher than in comparable months, or in any 3-month sequence, during the early period (Feldman et al. 1995). The tolerant 'I'iwi and the sick male 'Ākepa, while small in number, nevertheless are the first indications of both tolerance and morbidity at upper elevation. The spatially separated malaria-positive 'Elepaio indicate that several infectious mosquitoes occurred at our site. The following discussion centers on hypotheses besides temperature for the increase in malaria, climatological issues addressed by our study, and issues associated with tolerance and cost of tolerance.

#### ALTERNATIVE HYPOTHESES TO TEMPERATURE

There is little support for alternative hypotheses to explain the increase in malaria, as have been proposed for increases in human malaria at high elevations (Reiter 1988, Hay et al. 2002). There were no nearby land use changes between 1988 and 2001 that would have increased mosquitoes. The 33 000 acre refuge extends from 1000 m to 1940 m elevation, and surrounding lands of forest and pasture are little changed. Cattle were removed from the refuge in the early 1990s. Pig eradication on the refuge has decreased breeding opportunities for *Culex* mosquitoes by decreasing wallows and damaged tree fern trunks (*Cibotium* spp) that can collect water (Scott et al. 1986). Pools of water in the several gulches on the refuge could have provided breeding sites for mosquitoes, but the gulches have been unchanged by humans over the course of study. There were no vector control programs at any time.

Range expansion of *Culex* mosquitoes, independent of temperature, might have occurred. However, our detection of mosquito breeding at upper elevation is not the first. While no mosquito sampling occurred during the early period, mosquito eggs and larvae were detected over 70 years ago at elevations of 1981 m in an area 8 km north of our study site (Swezey and Williams 1932). More recent survey efforts on the refuge, which included our study site during 1993–1995 and 1997, suggested that mosquitoes were extremely rare and limited to a site 11 km north at 1585 m elevation during this time period (LaPointe 2000, Woodworth et al. 2001). Endan-

gered birds are much rarer or absent in the sites where mosquitoes were detected in these earlier studies, and mosquito-borne disease is a hypothesis for this geographical pattern (Scott et al. 1986). Our detection of mosquito breeding at our 1900 m site expands the range solidly into that of endangered bird habitat at upper elevation, in association with warmer air temperatures.

Increases in cases of host tolerance, which we documented, could also explain higher malarial prevalence observed, independent of temperature. Under this alternative, there would be no increase in malaria, just in numbers of birds that survived it to be tested. This hypothesis predicts that the species that tested negative for malaria during the early period and positive during the late period would increase in population size. The trend analyses for 'I'iwi and Hawai'i 'Elepaio do not support this hypothesis.

Also, there is the possibility of cycles of malaria at upper elevation and our early study coincided with a trough and our late study with a peak. However, the change in prevalence in the 'Elepaio makes this unlikely. The 'Elepaio colonized Hawaii from the Austral-Asian region at 0.43 to 1.06 mybp (Burgess 2005), much more recently than the equally old honeycreepers and thrushes (Lovette et al. 2002). Thus greater tolerance in this species is expected (Zeigler 2002). With its high annual adult survival (VanderWerf 1998), if no 'Elepaio tested positive during the early period, any previous peak would have preceded the early period by at least an 'Elepaio lifespan (10–12 years). Rarity of mosquitoes at upper elevation also argues against a cycle of malaria. An additional epizootic during another warm spell would provide a statistical link between temperature and prevalence that could be the basis of temporal variation in prevalence.

Finally, there is the possibility that it was improved methodology used during the later period that was responsible for the observed increase in prevalence. Better methodology would extend sensitivity into lower levels of parasitemia, and the improvement would be limited to these cases. However, a major epizootic, with mosquito breeding, warmer air temperatures, and sick and dead birds is unlikely to be composed of such low level infections. The lowering of parasitemia to the limits of detection by microscopy takes 2–4 months post infection (van Riper et al. 1986, Atkinson et al. 2000), and the epizootic

was within one month of the warmer air temperatures. Improved methodology may be more sensitive, but circumstances of the epizootic prevented the opportunity to detect many cases with very low parasitemia. Rarity of positive cases outside of the epizootic argues against chronic cases detectable only by the improved methodology.

#### CLIMATIC ISSUES

The issue for malarial transmission is the extent to which mosquitoes at upper elevation are infectious. Although the epizootic was associated with higher air temperatures, the run of 14 days during July–August with a mean temperature of 15.4°C was still not sufficient for sporogony to be completed in newly infected mosquitoes at upper elevations (LaPointe 2000). However, such warm spells could allow previously infected mosquitoes carried up from lower elevations to develop at least partial infectiousness to birds, and would increase mosquito development and biting frequencies (Harvell et al. 2002).

While our short-term weather data cannot address climate change, the major epizootic of 2001 portends grave consequences in climate warming scenarios. The existing model for Hawai'i projects a future temperature increase of 2°C that would eliminate most malaria-free bird habitat in Hawai'i over the next 100 years (Benning et al. 2002, Harvell et al. 2002). However, our measured temperatures already exceed future predictions because our site is strongly affected by the inversion layer derived from tropical air circulation. The inversion layer results in increases in air temperature with increasing elevation, the opposite of adiabatic lapse rate. Above the inversion layer, the normal adiabatic lapse rate returns, but is offset by the layer. As the inversion layer moves up and down, a fixed point on land will be below it some of the time and above it some of the time. The elevation of our site is the mean elevation of the inversion layer on Mauna Kea (Giambelluca and Schroeder 1998), so our site will be strongly affected.

The two existing models for studying the relationship between temperature and disease in Hawaiian birds simply use adiabatic lapse rate (Benning et al. 2002, Harvell et al. 2002). The 4.4°C difference between our measured summertime air temperatures (15.4°C), and those assumed by the current models (11°C), can be attributed to the inversion layer. The temperature

differential of the inversion layer has been estimated at a mean of 2.7°C  $\pm$  2.2°C SD (Tran 1995). The 4.4°C difference between assumed and observed is within one standard deviation of the temperature differential. This has enormous conservation implications, since the 13°C threshold for initiation of sporogony, predicted to occur at our study site in 80 years, is already exceeded. Perhaps the most important question with respect to climate warming and malaria in Hawaiian birds is what climate change will do to the inversion layer. The altitude of the base of the layer, height of the layer, and temperature differential in the layer are all climatic factors with implications for transmission of malaria.

#### TOLERANCE AND COST OF TOLERANCE

The two tolerant 'I'iwi, the first to be documented in the wild, reveal several issues associated with tolerance. Mosquito bites will have a variable number of sporozoites. Large numbers of sporozoites will achieve larger population size more rapidly than will small numbers. Small numbers thus give the bird's immune system more time to deal with the infection at an early stage. Our observations of tolerant 'I'iwi, and potentially the Hawai'i 'Ākepa, could be because the birds had mild infections. Alternatively, the strain of pathogen transmitted may have had reduced pathogenicity. Research is required for all host species to determine the threshold of sporozoite levels between tolerance and lack of tolerance, which may be expected to vary among species.

The broken head feathers in the two malaria-positive 'I'iwi illustrate difficulties in documenting a cost of tolerance. The feather condition could only have resulted from fault bars acquired during molt (Grubb 1989), followed by brushing the head against foliage while foraging, something 'I'iwi do hundreds of times each day. The issue is whether there is a trade-off between the activities of molting and maintaining an immune response. The basis for a trade-off is the intensity of infection. If the fault bars resulted from reduced foraging during intense illness while molting, then there would not be a cost of tolerance. In contrast, if the fault bars resulted from molting during a chronic infection, there would be a cost of tolerance. As the 'I'iwi went through a molt at least nine months following initial malaria detection, they would have been in a chronic phase during molt (van Riper et al.

1986), unless there was recrudescence. Barring recrudescence, a cost of tolerance is expected. However, the infection status of the bird while molting must be known to clearly identify a cost of tolerance evident in plumage.

Tolerant birds have two roles for studies of malaria. One role is that of sentinel for increased malaria at upper elevations. The Hawai'i 'Elepaio may be especially valuable because of its permanent residency. In addition, the bird forages lower in the canopy than most honeycreepers (LAF, pers. obs.). If roosting is also lower, then 'Elepaio will have greater exposure to *Culex* mosquitoes rising out of the understory at night. The second role of tolerant birds is as a hidden reservoir of disease. The parasite is already establishing its own residency, even when vectors are absent at upper elevations, through the reservoir provided by this multiple host community (Dobson 2004).

In conclusion, this study provides a preview of the risks of malaria for Hawaiian birds in upper elevation forests. It is a preview because models of increase in malaria transmission with climate warming do not predict epizootics such as we documented for many years into the future (Benning et al. 2002, Harvell et al. 2002). Moreover, unlisted species, as they form a larger reservoir of tolerant individuals, will contribute to malaria becoming an emerging infectious disease in endangered birds. Rare Hawaiian birds face greater threats from malaria than currently assumed. It remains to be seen if any endangered honeycreepers, with lower reproductive rates than unlisted species (Freed 1999), can evolve tolerance rapidly enough to avoid extinction. The vector and pathogen are also evolving, and the outcome may depend on rates of evolution in hosts, vector, and pathogen.

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