Studies in Avian Biology No. 22:264–273, 2001.

CHANGES IN NATIVE AND INTRODUCED BIRD POPULATIONS ON O'AHU: INFECTIOUS DISEASES AND SPECIES REPLACEMENT

CHERIE SHEHATA, LEONARD FREED, AND REBECCA L. CANN

Abstract. Bird species with their blood parasites have been introduced to the Hawaiian Islands in the last 150 years and alien bird species now outnumber native species in most lowland habitats. We conducted a survey of malarial prevalence in birds at one low-elevation site in urban Honolulu over a three-year period. In screening 311 birds (15 taxa) with a sensitive and accurate DNA-based diagnostic, we discovered that the average prevalence of avian malaria was about 10%, but that significant differences in prevalence existed among species at this site. Not a single case of malarial infection was detected in the 43 native birds, primarily O'ahu 'Amakihi (*Hemignathus flavus*), that were tested by polymerase chain reaction (PCR). It is well established that 'Amakihi (e.g., *Hemignathus virens*) on other islands are suitable hosts for *Plasmodium* strains present in Hawai'i, and that they survive at lower rates than introduced species when given malaria experimentally. Five introduced species have prevalence rates in excess of 20% and appear to be some of the primary reservoirs for maintenance of the disease among passerines on O'ahu. Five other introduced species showed no evidence of active malarial infections. Recaptures allowed us to discover that some alien species effectively cleared their parasites in 3 to 8 months after initially testing positive for *Plasmodium.* The survival of relict populations of native birds on O'ahu suggests that genetic resistance and/or tolerance factors to avian malaria are evolving; this is consistent with observations that 'Amakihi on other islands vary in their survival when experimentally challenged with malarial pathogens. In habitats where prevalence of malaria may be seasonal and at low levels, testing for avian malaria using blood smears is likely to underestimate the true impact of the disease. Successful management of honeycreeper relatives may now depend on identifying the genetic loci responsible for disease resistance, using the 'Amakihi model.

Key Words: Birds; disease; endangered species; Hawai'i; malaria.

The introductions of more than 125 alien bird species to the Hawaiian Islands since 1865 (Pimm 1991) and an appropriate mosquito vector in the early 1800s (van Riper et al. 1986) have had devastating consequences for native Hawaiian birds. As a group, native Hawaiian birds are now at about half the level of species diversity that existed 200 years ago, and half of these species are currently endangered (Freed et al. 1987a, Stone and Stone 1989). The extinction process of native species has accelerated with the rediscovery of the Hawaiian Islands by western explorers, such that the pattern of historical decline has been called bimodal (Ralph and van Riper 1985). This historical pattern parallels the near extinction of Native Hawaiian peoples to infectious diseases after the rediscovery of Hawai'i in 1778 (Stannard 1989), where models for virgin soil epidemics consider evolution in geographic isolation and modes of transmission to be important features for predicting changes in pathogen virulence (Ewald 1994).

The Hawaiian Islands have more alien bird species than any other place on earth, and most of these species have been introduced since 1893 (Pratt 1994). The islands were once geographically remote, extremely isolated habitats, even for birds (Olson and James 1982a). That status changed with the arrival of Polynesian and European explorers, who brought with them a host of predators, pathogens, and avian competitors (van Riper et al. 1986). The association of the current decline in native bird populations with the continued introduction of alien species is attributable to at least two hypotheses: (1) direct competition between natives and aliens for food, nesting, or other resources (Moulton and Pimm 1983); and (2) greater susceptibility (morbidity, mortality) of natives to infectious diseases and novel strains of pathogens that arrive with each introduction (Warner 1968, van Riper et al. 1986, Atkinson et al. 1995, Cann et al. 1996).

There is little direct evidence to support the first hypothesis because habitats with ecological variables that appear suitable for native birds are sometimes completely devoid of them (Scott et al. 1986, Freed and Cann 1989). Steep distributional gradients now mark the ranges of many endangered species on the high-elevation islands as if there were some invisible but deadly force restricting species recovery though their habitats are now protected.

The continued decline during the last 30 years of native birds in low-elevation forests on the island of O'ahu (Williams 1987, Pratt 1994) also is especially problematic. These are the habitats of native species most accessible to educators

and their students, naturalists, ecotourists, and policy makers. Yet these forests have few native birds. Remnant populations of less than 1,000 individuals spread over 32 km may not represent truly viable groups. One thinks immediately of the case of the O'ahu 'Elepaio (*Chasiempis sandwichensis ibidis*), a territorial species with disjunct populations isolated on two separate mountain ranges (VanderWerf et al. 1997).

Declines in the absence of habitat degradation or obvious competitors and predators are consistent with the disease hypothesis. All Hawaiian forests have alien bird species, and the distribution of native birds is generally limited to elevations where the introduced *Culex* mosquito is rare (van Riper et al. 1986). Alien birds are linked to habitat loss, predation, competition, and introduced diseases, all of the major factors thought to account for the wave of extinctions between 1893 and 1910 (Ralph and van Riper 1985). It is also possible that the alien birds introduced since 1910 pose an even greater disease threat to native birds than previously thought. Newly introduced organisms can bring with them novel pathogens and may also acquire the parasite faunas of resident species, altering disease transmission patterns. Alien vertebrates can even reduce their parasite load upon translocation to new habitats (Lewin and Holmes 1971), especially if intermediate hosts are lacking in the new environment or dietary changes accompany the shift in range.

In the midst of all these difficulties, isolated pockets of native birds exist in low-elevation forest habitats on at least two Hawaiian Islands, where mosquitoes are present year-round in high densities and researchers suspect there are very high rates of *Plasmodium* infection (Scott et al. 1986). We have identified such a population of honeycreepers in the O'ahu 'Amakihi (*Hemignathus flavus*) at Lyon Arboretum, a protected, second-growth forest habitat that is affiliated with the University of Hawai'i on the island of O'ahu. We have a special interest in this study site, because it is also the focus of native plant restoration attempts and, as such, is an important resource in the battle to conserve tropical biodiversity (Turner and Corlett 1996).

Only two species of native honeycreepers, the O'ahu 'Amakihi and the 'Apapane (*Himatione sanguinea*), are present at Lyon Arboretum. The 'Amakihi is a year-round resident, while the much rarer 'Apapane is usually found only when floral resources are abundant in the arboretum and low to absent elsewhere. In contrast, at least 30 alien species of birds are normally sighted at the location, some of them escaped exotics from an adjacent tropical garden. These alien species are known to host a variety of parasites (van Riper and van Riper 1985).

Birds living in the arboretum, which has been reforested with a mixture of exotic tree species since the 1920s, are also potentially coexisting with a variety of disease vectors. Any native bird surviving in this habitat has experienced more than 70 generations of breeding in association with multiple vectors, parasites, and reservoirs. Native birds in this habitat are therefore prime candidates for evolving genotypes tolerant or resistant to malaria. The exotic bird species included in this study and the estimated date of their introduction to Hawai'i are shown in Table 1.

We previously devised a PCR-based test that was capable of detecting malarial infection in many species of passerine birds. The test used a 50 μl blood sample taken during the mist-netting and banding of birds (Feldman et al. 1995). Data from this test documented the presence of malaria in high-elevation zones previously thought to be safe habitats for native birds, but the assumption of safety was based on only limited knowledge of the dynamics of the disease in low-elevation habitats containing large numbers of introduced birds and mosquitoes (Cann et al. 1996). An extensive survey of malarial prevalence at the Lyon Arboretum site was therefore initiated to more accurately estimate the true importance of this disease for bird populations living in relict, lowland Hawaiian forests and to help address the continued decline of native birds on O'ahu.

METHODS

Birds were caught in pole-based and aerial mist nets using standard ornithological methods and following all animal safety regulations. Blood samples were taken from birds by puncturing the wing vein with a sterile 26-gauge needle. A total of 311 individual birds were bled and examined visually for signs of ectoparasites and poxlike lesions. Each bird was tagged with a unique color band and/or a standard aluminum identification band and was measured, photographed, and released.

Approximately 50 μl of blood was withdrawn as per Feldman et al. (1995), and total genomic DNA was prepared using the low-volume modification method of Quinn and White (1987b). Amplification of a fragment of the 18s rRNA gene from either the disease agent or the bird was performed and scored as in Feldman et al. (1995). All birds were tested in at least two separate amplification reactions, with appropriate extraction and with positive and negative controls. Only unambiguous birds were scored in this test, with the 18s rRNA gene fragment of the host bird's cell serving as an internal control for successful amplification.

Infectious state (positive or negative) was analyzed using a logistic regression with status (native or introduced) as a class variable and species within status as a nested variable. Chi-square tests were derived from

TABLE 1. MALARIA FOUND IN BIRDS SAMPLED AT LYON ARBORETUM, HONOLULU, HAWAI'I, FROM 1994 TO 1996

Species	Place of origin	Time introduced	Number Positive	Total	Percentage
Native Honeycreepers					
O'ahu 'Amakihi			0	42	0
'Apapane			0	1	0
Total			0	43	0
Introduced species					
Common Myna	India	1879	0	2	0
Common Waxbill	Africa	early 1900s	1	36	2.8
House Finch	North America	1800s	0	3	0
Java Sparrow	South-east Asia	before 1965	0	10	0
Japanese White-eye	East Asia	1929	4	87	4.6
Northern Cardinal	North America	1929	0	6	0
Nutmeg Mannikin	India	1865	6	27	22.2
Red-billed Leiothrix	South Asia	1918	1	19	5.2
Red-vented Bulbul	India	1965	1	2	50
Red-whiskered Bulbul	India	1966	11	40	27.5
Spotted Dove	South-east Asia	1800s	1	5	20
White-rumped Shama	South-east Asia	1940	6	26	23.1
Zebra Dove	Australia	1922	0	5	0
Total			31	268	11.6
Sample Total			31	311	10

the generalized linear model functions of S-Plus (Venables and Ripley 1994).

RESULTS

Native birds had significantly lower prevalence of malarial infection than did introduced birds ($P = 0.002$; Table 1). In fact, none of the native birds tested positive despite being sampled at the same time and place during which introduced birds tested positive. The species screened and numbers of malaria positive individuals identified during the three-year period (1994–1996) are listed in Table 1. Eight of the 15 species tested were found to be infected with the pathogen.

There were also significant differences in prevalence rates ($P = 0.002$) among the species of introduced birds (Table 1). The highest rates of malaria (>20%) were found in White-rumped Shama (*Copsychus malabaricus*), Red-whiskered Bulbul (*Pycnonotus jocosus*), Red-vented Bulbul (*Pycnonotus cafer*), Nutmeg Mannikin (*Lonchura punctulata*), and Spotted Dove (*Streptopelia chinensis*). Five species of introduced birds that were free of malaria at the time of testing were Common Myna (*Acridotheres tristis*), House Finch (*Carpodacus mexicanus*), Java Sparrow (*Padda oryzivora*), Northern Cardinal (*Cardinalis cardinalis*), and Zebra Dove (*Geopelia striata*). Low, but non-zero, rates of infection were observed in Common Waxbill (*Estrilda astrild*), Japanese White-eye (*Zosterops japonicus*), and Red-billed Leiothrix (*Leiothrix lutea*). Malaria affected 12% of the birds that were screened in 1994, 9% in 1995, and less than 9% in 1996 (Fig. 1). Overall prevalence of malaria at the study site for the three-year period was 10%.

DISCUSSION

WHAT ABOUT THE SICK BIRDS THAT CAN'T FLY?

It is possible that we failed to find native and alien birds on O'ahu infected with malaria because sick birds do not normally fly into mist nets. We never encountered dead or moribund birds in the forest during our hours of mist-netting, but the overall probability of such discovery is low. A single 'Apapane has been discovered near death by arboretum staff at this site in the last six years of our working there. We simply cannot say that our sample of mist-netted individuals represents an adequate survey of all birds present in the habitat. Perhaps all native birds contract malaria, and the only ones well enough to fly are those with immune systems capable of clearing the parasites to tolerable levels that are below our ability to detect them with current techniques.

We can, however, evaluate the likelihood that our total sample was insufficient to discover infected birds across the board by reference to standard epidemiological modeling. A sample size of 246 individuals is sufficient to estimate within 5 percentage points the true value of disease incidence, with 95% confidence, if the value of the true rate is unlikely to exceed 20% (Lwanga and Lemeshow 1991:25). Our sample of 311 birds therefore appears to have been sufficiently large to have uncovered infected birds,

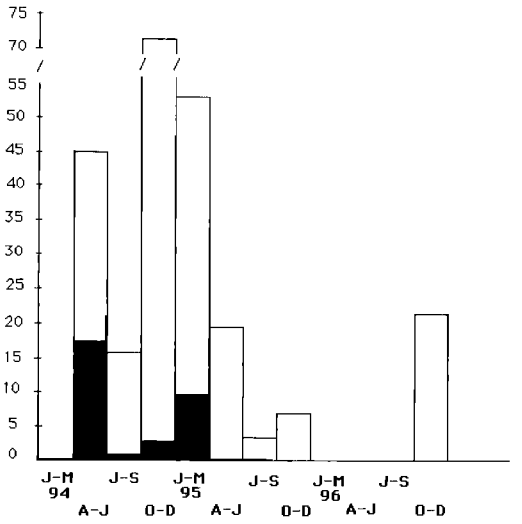

FIGURE 1. Capture history of diseased birds over the 1994–1996 study interval. Data for the eight species showing malaria are grouped in 3-month intervals, with total number of birds captured in any interval shown on the y axis and the number of diseased birds in that sample indicated by shading. J–M = January, February, and March; A–J = April, May, and June; J–S = July, August, and September, and O–D = October, November, and December. The number under the first quarter indicates the year of the sample for the following intervals. O'ahu 'Amakihi were captured in each.

if they truly existed, over the study period. If there is a general prevalence of malarial infection of 10%, and 14% of the total sample were native birds, it is somewhat unusual that not a single native bird was scored positive, given our knowledge of their susceptibility to this pathogen when experimentally challenged.

Additionally, the possibility that native birds were differentially affected at the nestling and fledgling stages, where their decreased mobility or immature immune systems might render them even more susceptible to parasitic infection (Ricklefs 1992), can be addressed. Of the 42 'Amakihi sampled, 4 were hatch-year birds, 6 were second-year birds, and 32 were after hatch-year birds. We therefore expect that some fraction of the 10 youngest 'Amakihi in this sample might still have circulating evidence of a past malarial infection, because other studies have tested how long after a deliberate inoculation with *Plasmodium* that a PCR signal can detected. C. Atkinson and C. van Riper (pers. comm) have followed experimentally challenged birds and estimate that the PCR detectable sequence is present for a year post-infection.

Challenge experiments with adult native 'Amakihi after a single bite from an infected mosquito show that an acute stage of parasitemia develops within 10 days, killing approximately 60% of the birds within 3 weeks of infection (Atkinson et al. 2000, Jarvi et al. *this volume*). Birds surviving this challenge show PCR-positive results for up to a year after infection, though some of these experimentally infected Amakihi are PCR negative (C. Atkinson, pers. comm.). Thus, it is likely that if 'Amakihi on O'ahu are easily infected, our sample of juvenile and young individuals should have contained a few birds still harboring enough parasites to render them PCR positive. All 42 'Amakihi that were tested appeared free of infection, consistent with their local evolution of genetic characteristics rendering them tolerant or resistant to malaria. Affected individuals of eight alien species were present in the same habitat (Fig. 1) during the same time that 'Amakihi were tested, showing that the vector is prevalent, and the 'Amakiki are known to be year-round residents of the area.

The Relevance of the 'Amakihi Model

We infer that the O'ahu 'Amakihi population sampled in this study has evolved some mechanism(s) of genetic resistance that now allows it to survive in lowland forests where malaria is prevalent. This hypothesis is consistent with the observation that 'Amakihi populations from the island of Hawai'i contain individuals capable of surviving experimental challenge with malaria (van Riper et al. 1986, Atkinson et al. 2000). The 'Amakihi populations of O'ahu may be similar to the case reported for the New Zealand Bellbird (*Anthornis melanura*), where recovery is apparently unrelated to a decline in predators or a decline in habitat destruction (Steadman et al. 1990). If the adaptive radiation of Hawaiian honeycreepers truly began less than 10 million years ago (Johnson et al. 1989, Tarr and Fleischer 1995), the 'Amakihi population sampled here represents the Rosetta Stone for potentially manipulating the genomes of many endangered honeycreepers, with whom they share most of their evolutionary history.

'Amakihi are small nectivores/insectivores that exhibit some aspects of territorial behavior during breeding. Their nesting and foraging behaviors are well documented (van Riper 1987), and they have become important surrogates for research into captive propagation of endangered Hawaiian honeycreepers (Kuehler et al. 1996). 'Amakihi are generalists that exist in large numbers on the islands of Kaua'i, Maui, and Ha-

wai'i, and are considered one of the most adaptable of the remaining honeycreeper species (Scott et al. 1986). Populations on the older islands of O'ahu and Kaua'i are accorded separate species designations, whereas Maui and Hawai'i populations are considered separate subspecies (Pratt et al. 1987).

We investigated the genealogical relationships between 'Amakihi using mitochondrial DNA (mtDNA) sequences from the cytochome *b* gene to explore whether there were deep phylogenetic subdivisions that might restrict the utility of classic genetic mapping for disease loci in this genus. Sequences were amplified from total genomic DNA using conserved primer sequences as in Kocher et al. (1989), and we used additional sequences as in Feldman (1994). Based on an alignment of approximately 220 nucleotides of cyt *b* for 68 birds, we confirmed that O'ahu lineages form a separate island group of maternal genealogies in the genus, and we have identified at least five separate maternal lineage groups that currently exist in 'Amakihi using distance, parsimony, and likelihood clustering methods (Cann and Douglas 1999). We estimated from this study that the coalescence of the mitochondrial genome for the O'ahu sample was approximately 300,000 years ago, based on an assumption of a crude rate of substitution at 2% per million years for the cyt *b* gene as a whole (Irwin et al. 1991).

All O'ahu birds tested negative for *Plasmodium,* so presumably the mutations conferring tolerance or resistance arose in a common ancestral lineage, but this might be shared with the Kaua'i population. Maui and Hawai'i 'Amakihi lineages belong to a different set of maternal genetic lineages; therefore, we suspect that the ability to tolerate or resist malarial pathogens has arisen independently in these birds since the introduction of the mosquito as an appropriate vector.

The Dynamics of Disease Transmission

In Hawai'i, the disease relationships between native and introduced bird species are by no means clear. Native birds are known to show a higher degree of susceptibility to malaria when exposed experimentally, and they have more severe infections than introduced species (Warner 1968, van Riper et al. 1986, Atkinson 1995). This pattern fits the virgin soil model, where initial exposure can result in the loss of up to 95% of the host population in as few as two generations. Stochastic factors (Lande 1988) might then finish off the remaining population, as fragmented groups suffer from highly skewed sex ratios and loss of behaviorally experienced individuals. Twenty years of monitoring infectious disease in Hawaiian birds has convinced most conservationists that malaria is a major factor limiting the recovery of native forest bird populations below 1,800 m (van Riper et al. 1986).

Researchers now generally considered that only a single species, *Plasmodium relictum capistranoae,* is currently infecting bird populations (Laird and van Riper 1981), and that a single species of *Culex* mosquito is primarily responsible for the transmission of this pathogen. Unfortunately, *Culex quinquefasciatus,* is abundant year-round on O'ahu in habitats below 1,600 m. The mosquito is also capable of transmitting avian poxvirus, a disease that can cause blindness by secondary bacterial infections and can inhibit the ability of perching birds to forage efficiently due to the loss of digits (see also VanderWerf *this volume*). As predicted by the disease model, the distribution of native birds on most islands is inversely related to the density of mosquitoes (Scott et al. 1986).

Various authors commenting on avian extinctions have speculated about a potential role the introduced bird community may play in serving as a disease reservoir (van Riper et al. 1986, Steadman et al. 1990, Pimm 1991, Feldman et al. 1995), especially given the continued decline of native birds on O'ahu following analysis of the Audubon Society's Christmas Bird Count (Williams 1987). At least 22 new species of birds were recorded on O'ahu during the 1960s (Moulton and Pimm 1983), and at least some of these introductions resulted in the establishment of new breeding populations.

The Red-whiskered Bulbul, introduced to O'ahu in 1966, shows a prevalence of 27.5% malarial infection over the period of this study, consistent with its potential to act as a potent source of pathogens in the resident bird community. Of the species with prevalence rates in excess of 20%, however, Nutmeg Mannikins (22.2%) and Spotted Doves (20%) represent older introductions to Hawai'i from India and Southeast Asia in the 1800s, and five introduced species (including some recently introduced taxa) were completely free of infection. New studies should now focus on strain identification of the pathogens and their associations with particular species, in order to address the question of recently introduced species and more virulent pathogen genotypes.

The hypothesis of alien species-as-disease reservoir was also deemed less likely by the discovery that native birds on the island of Hawai'i are commonly infected with malaria in low-elevation forests (Atkinson et al. 1995). This finding showed that the native populations are capable of maintaining their own disease reservoir and has led some researchers to discount the im-

pact introduced species have had on the continuing disappearance of native forest birds. Our results documenting a higher level of malaria in alien birds on O'ahu suggest that the ecology of disease transmission may be different on the two islands, perhaps because the communities of native and introduced species differ in their exact makeup (Pratt et al. 1987).

Infection Versus Disease and Mechanisms of Disease Resistance

Genetic loci implicated in resistance to malaria are often members of the major histocompatibility complex (*Mhc*), a supergene family containing sequences important in presenting fragments of degraded molecules to cells of the immune system (see, e.g., Jarvi et al. *this volume*). The family also contains complement, collagen, proteasome-like, transporter, cytokine, and heat shock genes (Klein 1986, Trowsdale et al. 1991, Hughes and Nei 1992, Klein and O'hUigin 1994). The bird model for the *Mhc,* the domestic chicken, has duplicated the *Mhc* regions (B@ and RFP-Y@) on two ends of microchromosome 16 (Fillon et al. 1996). So far, no association has been made between particular *Mhc* loci and resistance to malaria in birds (Stevens 1996).

Resistance to or tolerance of malaria, however, cannot be understood simply from the perspective of the *Mhc.* Owing to the fact that malarial infections involve a parasite that cycles between sexual and asexual life phases, uses several hosts, and undergoes rapid change in surface antigens presented to the host, it is possible to control its proliferation at many points. Natural resistance to malaria in humans has also been linked to Duffy blood group antigens, glucose 6-phosphate dehydrogenase variants, sickle-cell hemoglobins, alpha and beta thalassemias, and various transport proteins, as well as the *Mhc* class 1 and 2 genes (Weiss 1993).

Much of the epidemiological evidence associating a particular *Mhc* haplotype or variant with disease resistance to malaria is actually indirect (Mascie-Taylor 1993), and of a questionable experimental nature owing to systematic underestimates of the prevalence of malaria in well-studied populations (Bottius et al. 1996). In mice, natural immunity to malaria appears to be linked to a non-*Mhc* major gene (Malo and Skamene 1994) with contributions from other loci. Thus, resistance to malaria should be treated as a quantitative genetic trait, and it may be misleading to search only among the 'Amakihis *Mhc* for variants conferring natural immunity to *Plasmodium* parasites.

Birds that can tolerate a certain number or strain of parasites, because of genetic factors, may be capable of harboring a *Plasmodium* infection but never show clinical symptoms of malaria. We are therefore incapable of stating at this time that birds scored as infected using the PCR test have now or have had in the past full-blown malaria. Atkinson's followup of our PCR negative samples by serological tests indeed identified an O'ahu 'Amakihi with immunological evidence of past infection (C. Atkinson, pers. comm.). Studies like the one performed by Hulier et al. (1996), which follow the development of parasites in infected organs of the host animal, will be necessary to differentiate between these two states. Animals that survive infection, however, might serve to illustrate the first stage of adaptation and be used as models for illustrating different levels of genetic resistance.

Dobson and May (1986) have shown that the major factor in the time it takes for a native host population to evolve a significant degree of genetic resistance to an introduced pathogen is the cohort generation time of the host species, and that initial frequency of the resistance gene, gene dominance, or strength of selection for resistance (and therefore fitness of both heterozygotes and homozygotes) affect resistance time only in a logarithmic fashion. Resistance typically arises in 5 to 50 generations. 'Amakihi are capable of breeding within six months of hatching (van Riper 1987), so it appears that an appropriate length of time has elapsed for natural selection to have resulted in the evolution of resistant genotypes to certain infectious diseases in Hawai'i, assuming native birds are breeding *at minimum* on an annual cycle. Epizootic malarial transmission in Hawai'i probably began sometime between the 1826 introduction of a suitable vector (Warner 1968) and the decade beginning 1870 when Skylarks (*Alauda arvensis*), Spotted Doves, Common Mynas, and House Sparrows (*Passer domesticus*) appeared on O'ahu. Thus, a minimum of 170–125 generations has elapsed for natural selection to result in the evolution of resistant genotypes.

Our finding that at least three species of introduced birds were capable of clearing malarial infections in 3–8 months, based on recapture data, is also consistent with the hypothesis that introduced birds coevolved in their native ranges with the *Plasmodium* pathogens for a longer period of time, and that they now contain greater numbers of individuals in their populations with malarial-tolerant genotypes. Immunity to malaria is generally strain specific, may be stage specific as well, and can also entail a number of cellular mechanisms that help limit the life cycle of the pathogen (Wakelin 1996). A host may have the ability to restrict or modify the move-

ment of *Plasmodium* parasites during invasion of cells, or can prime the synthesis of additional cytokines, helper T cells, or other mediators. In addition, the host may be able to prevent binding of the parasite to vessel endothelia, or to neutralize the toxins produced when schizonts rupture host cells. A host may also have the ability to control the reproductive stage of the parasite.

Any genetic mutation in the host genotype affecting the growth of one strain or species of *Plasmodium* during infection does not necessarily confer immunity to another strain. Resistance/susceptibility may be due to primarily *Mhc*-T cell interactions for one strain and B cell factors for another. Molecular studies can eventually map all the loci contributing to resistance in each species of bird, but these features underscore the need to search beyond *Mhc* loci for genetic resistance/susceptibility to malaria.

Implications for Management and Recovery

van Riper and van Riper (1985) drew attention to the continuing threat of disease to bird populations of Hawai'i, and the role that management must play in monitoring and vector control. Nothing has changed since that report cataloged the known avian disease pathogens of Hawai'i and their hosts. Captive and domesticated birds continue to be imported, as well as captive-bred native species, like the Hawaiian Goose (*Branta sandvicensis*), or Nēnē. Stepped-up efforts at captive rearing of the goose and the Hawaiian Crow (*Corvus hawaiiensis*), or 'Alalā, have resulted in more stringent quarantine protocols at rearing facilities, but game birds, natives, and exotics from around the world continue to mix in our forests and in mosquito-laden zoo environments. It appears that arboviruses, Newcastle disease, and avian influenza have still not made it to Hawai'i, and extreme care is necessary to maintain this condition, especially now that animal quarantine regulations have been relaxed. If anything, the threats and problems caused by infection and disease (Scott et al. 1988) have increased in magnitude, with the rediscovery of tiny populations of some endangered species.

Prior to our analysis, the only comprehensive study of disease pathogens in the introduced bird populations on O'ahu examined 121 individuals from 21 species (Smith and Guest 1974). That study documented protozoan infections (*Coccidia* and *Trichomonas*) in 20 birds and found evidence of helminths infecting 40, but it did not identify malaria as a significant component of the parasite load in these species. The site of study in this instance was the western slope of Diamond Head, a significantly dryer habitat than the arboretum in the Manoa Valley where we worked. It is possible the malaria was not a significant disease at that time, but it is more probable that limited resources did not allow a full exploration of potential disease pathogens, and that the level of parasitemia may have been too low for detection by classical blood-smear methods.

The full impact of malaria on bird populations can only be evaluated with an efficient diagnostic that can detect very low levels of the parasite. The reported absence of protozoan parasites in the Cook Islands (Steadman et al. 1990), attributed to a very low level of prevalence in native and colonizing species, may actually be due to inadequate methods of detection using diagnosis by blood smears. We suggest that no population be considered *Plasmodium*-free unless PCR-based diagnostics are employed. If disease prevalence is not measured accurately, continued discussion about characteristics of successful invasions (e.g., Pimm 1991) in Hawai'i and elsewhere is likely to omit crucial pieces of data. Most ecologists sample their systems in coastal forests below 610 m in elevation. In Hawai'i, such low-elevation sites are highly degraded and are usually characterized by a mixture of predominantly introduced species. Our suggestion that the disease hypothesis more fully accounts for the continued decline of native birds, rather than the effects of competition between introduced and native species, is based on our findings that malarial infections have been systematically underreported in all species tested prior to PCR-based assays. (A more complete discussion of these issues can be found in the appendix to this paper). What remains to be examined is the direct role of malaria in limiting survival and recruitment, using populations of banded birds and continual monitoring over several annual cycles of reproduction.

Captive rearing efforts using Hawai'i 'Amakihi (*H. virens*) as a surrogate species for studies of rearing, release, and restoration of endangered birds have been unsuccessful to date, because all birds reared succumbed to massive *Plasmodium* infections after their release (Kuehler et al. 1996). Eggs were taken from nests for hand-rearing from an area where the population was known to be highly susceptible to malaria (van Riper et al. 1986). If eggs are chosen with more attention to disease characteristics, it is likely that post-hacking survival will increase.

One can anticipate pressure to exhibit captive-reared native honeycreepers to the public, in order to justify the extraordinary expenditure of resources aimed at preserving a few endangered species. However, this action needs to be weighed against the relative risks of introducing novel pathogens to the remaining native bird

community, which is the predicted result of aviculture of many species in a common rearing environment. New molecular methods to designate birds disease-free should be supported by specialists in captive rearing and employed to screen potential candidates for either exhibition in nonmosquito proof cages or release into the wild.

Translocation studies of native birds into and between forests within the mosquito zone should consider the probability of enhanced long-term survival by the judicious choice of individual birds, especially if variation in natural immunity to mosquito-borne diseases exists among target species. If important factors (sex, age, appropriate genetic markers, vocal patterns, nest-building behaviors, plumage variation) are otherwise balanced, it seems wise to begin these translocations with birds that have a better possibility of tolerating malaria. Reuse of nest sites can also increase the probability of disease transmission (Loye and Carroll 1995), so some obligate cavity nesters may be more vulnerable to these dangers upon translocation.

Emergence of more virulent pathogens is one potential outcome of enhancing the frequency of resistant hosts by both natural and artificial selection (Ewald 1994). Management decisions that result in incomplete removal of parasites, through baited pharmaceuticals, may fail due to the inability to control drug dosages in natural populations of free-flying birds and will likely be counterproductive. We therefore cannot advocate treating malarial infections in endangered bird populations by offering them food items laced with antimalarials. Until effective vaccination is possible, vector control efforts offer the only sure route of breaking epidemic disease cycles.

Removal of certain alien bird species known to harbor malaria in critical habitats of highly endangered birds is a last resort. Such management action is likely to be a stopgap at best, but for alien species known to have sedentary or territorial behaviors placing them in direct competition with resident natives it may work as a short-term strategy, especially if vector numbers are low or fluctuate seasonally. To be effective, this action would depend on baseline knowledge of seasonal disease prevalence in the bird populations, some degree of geographic isolation between habitats, and effective year-round monitoring for infection.

CONCLUSIONS

This study documents a new, hopeful outlook on malaria as a factor limiting the recovery of native Hawaiian bird populations in urban, lowland habitats. Native birds coexisting with malarial pathogens represent individuals with genotypes that have effectively solved one infectious disease problem. Molecular markers offer us the opportunity to identify the genetic loci responsible for natural immunity, and to boost the numbers of individuals carrying these loci in natural populations. O'ahu 'Amakihi populations should be examined for DNA markers of disease resistance and used as the sires and dams in captive-rearing experiments. Long-term maintenance of genes for disease resistance or tolerance may require a metapopulation to be operating that geographically structures resistance genes and allows cycles of local population extinction with recolonization (Thompson 1996). This can be enhanced by judicious translocation and captive rearing.

ACKNOWLEDGMENTS

This study was funded by a grant from the John D. and Catherine T. MacArthur Foundation, World Environment and Resources Program. Support for this research was also provided by a grant from the Howard Hughes Medical Institute (HHMI) through the Undergraduate Biological Sciences Education Program. C. Shehata was an HHMI Undergraduate Research Fellow at the time of this study. We thank C. Atkinson, G. Massey, C. van Riper, III, B. Nakamura, L. Goff, D. La Pointe, M. Burt, J. Rohrer, E. VanderWerf, D. Haderman, M. Ono, K. Fernandez, D. Tupper, L. Douglas, and J. Maag for their samples, help, and discussion. An anonymous reviewer greatly improved this paper.

APPENDIX.

Effectiveness of This Screen for Detection of Infected Birds

A. Is the PCR test reliable?

Colleagues have asked us to address the question of reliability for the diagnostic employed here, compared to traditional smear-based methods involving light microscopy that they are more familiar with. They fear we have an unknown rate of false positives, as well as false negatives, associated with our PCR-based test, and that the high rate of infection seen for some alien species is an experimental PCR artifact. We can report on our own experience with this type of comparison, as well as relay data that exist in the literature for screening of subpatent malarial infections in humans, lizards, and rodents. There is general agreement among specialists in the field that PCR-based tests are preferred to smear diagnostics when parasitemia levels are low, when mixed infections are present, and when new dipstick-style tests are to be evaluated (Humar et al. 1997).

Information was already presented by Feldman et al. (1995) showing that, in two blind samples of Hawaiian birds, the PCR test using appropriate 18s rRNA primers correctly identified all birds judged infected by smear diagnostics. What we did not report in that publication was the finding that the PCR positive birds we identified, but which were not found initially by smear tests to contain *Plasmodium* infected cells, were later

reexamined with additional effort and found to be indeed infected (C. Atkinson, pers. comm.). We felt this was evidence that the assay was indeed more sensitive than we could measure adequately at the time, because DNA extracted from avian blood contains primarily the host DNA from nucleated erythrocytes, which could have reduced the efficiency of PCR amplification to target the parasite gene.

This comparison allows us to have greater confidence in the assertion that low-levels of infection sufficient to give a positive PCR result are often missed on blood smears for reasons that can range from time and effort of the slide reader to the physiological sequestering of parasites during certain stages of infection. It is estimated that roughly 200 times more blood cells are assayed in the PCR test than would be counted in microscopic fields (Snounou et al. 1993), and a study specifically designed to examine subpatent infections in humans estimates that in total sensitivity, PCR is 100 to 1,000 times more sensitive than microscopy (Bottius et al. 1996). A comparison of PCR with nested primers versus smear efficiency in western fence lizards (*Scleroperous occidentalis*) infected with the parasite *Plasmodium mesicanum* found that the more sensitive nested PCR easily detected very low-level infections, those scored as <1 parasite per 10,000 erythrocytes (Perkins et al. 1998), and this is also the conclusion of a second comparison (Khoo et al. 1996) with *Plasmodium falciparum* in humans.

Following the publication of an early PCR-based test using ribosomal primers for human *falciparum* malaria (Barker et al. 1992) different from the ones employed here and based on different cycling parameters, it was suggested that false positive rates of 16% and false negative rates of 5% were associated with the PCR method (Weiss 1995). False positives result from nonspecific priming of ribosomal gene families and their pseudogenes, and this can be controlled by better choice of primer sequences as well as nested primers in a two-step test. False positives can also be the result of sample contamination, which can be managed by good laboratory practices and is easily detected with appropriate positive and negative controls. False negatives are based on the failure of signal to amplify, either because some reagent is faulty, cycling parameters are not optimum, or due to stochastic effects with low parasite target numbers during early amplification stages. All these problems can be addressed by appropriate controls and multiple PCR runs on the same samples.

On the surface, a rate of 16% for false positives appears alarmingly high. However, this must be compared to an even higher but as yet largely uncalibrated rate of false negatives associated with classical smear tests. For human malarial diagnostics where correct drug treatment places high demands on testing accuracy, values in the literature range from 9% to 67%, reflecting a variety of field and laboratory conditions encountered by biologists (Kain et al. 1993, Ntoumi et al. 1995, Bottius et al. 1996, Khoo et al. 1996).

Snounou et al. (1993) demonstrated that correctly performed PCR can achieve an absolute, i.e. all or none detection accuracy, when titrated against controls (infected, cultured cells). Additionally, one quantitative study of malaria parasite development in mice has suggested that PCR methods previously criticized as inaccurate correctly predict infections when as few as 500 injected sporozoites are followed by a variety of quantitative biochemical measures, with implications for vaccine development (Hulier et al. 1996). In the lizard study cited previously (Perkins et al. 1998), it was found that false negatives (those samples scored as not infected after the blood smear, but found infected via PCR) were approximately 5%, but that a greater proportion of infections was detected only by PCR at a site deliberately chosen to study low prevalence transmission dynamics, where malarial infections were averaging about 6% of the total population (50% versus 9%). This was also the conclusion of the study of transmission dynamics with human malaria in Malaysia (Khoo et al. 1996). Under conditions where 50% of the infections in a population are characterized by low parasite counts, rare transmission appears to select parasite genotypes that sustain low parasitemia, as predicted by Ewald (1994). Decreasing parasite loads also result in a generally more complex genotypic array of parasites sequestered in the hosts body (Ntoumi et al. 1995). Given this information, it is clear that PCR technology brings many advantages, increasing our understanding of malarial epizootics in Hawaiian birds.

We should sensibly adopt the general position that sensitivity of both PCR and smear methods to correctly detect infection decreases as the number of parasites decreases. Evaluation of various diagnostic techniques under low levels of infection now posits that microscopy has an 83–86% sensitivity rate compared to PCR (Humar et al. 1997, Pieroni et al. 1998). Even with high sensitivity, birds with *Plasmodium* may be infected but may not suffer from malaria, emphasizing the importance of long-term studies on banded populations of birds where recaptures can be evaluated for disease status. As a rule, all birds should be screened at least twice by a PCR-based test in order to be considered free of *Plasmodium.* In addition, immunological tests that assay for the presence of past infection through western blot technology (Sambrook et al. 1989) have revealed that at least one of our 'Amakihi samples that was scored as negative by PCR showed evidence of antibodies to erythrocytic stages of *P. relictum* (C. Atkinson, pers. comm.). This clearly reveals that a PCR negative bird may have a negligible parasite status at the moment, but it may be impossible to say that a bird was never truly infected by a parasite, only that it is capable of mounting an immune response that limits infection.

B. Under what conditions might extra (>2) amplification products be produced?

Aside from known problems associated with failure to optimize PCR reactions involving low annealing temperatures, unbalanced deoxynucleotide ratios, extra cycling steps forcing carryover products, contamination by human cells, and magnesium concentrations, there are other factors that may lead to the occasional appearance of more than the two fragments expected in the Feldman test. An obvious one is that organisms undergo mutational change, and divergence in genetic sequences can be due to both length changes as well as substitution changes. If two pathogens coexisting in the same host were to undergo sexual recombination,

their genetic sequences might represent a new combination of information not previously seen in either parental strain. The new pathogen might have a gene fragment longer or shorter than the one expected on the basis of sequences currently found in Genbank.

Humans are commonly infected with multiple genetic strains and species of malarial pathogens, and the genetic characteristics of these strains change over the course of an infection (Ntoumi et al. 1995). *Plasmodium falciparum* is known to harbor six sets of rRNA genes per haploid genome (Rogers et al. 1995) that are expressed in stage specific manner (McCutchan et al. 1995). Judging from this example, birds in the wild probably contain an unknown number of different strains and/or species of pathogens, and to date no systematic molecular analysis of pathogen species diversity has been undertaken for Hawaiian birds. Additionally, *Plasmodium* contains an obligate plastid-like organelle (Kohler et al. 1997) with its own 35 kilobase circular genome containing multiple ribosomal sequences. There remain important questions to explore involving the use of ribosomal genes to identify species, but ribosomal genes are usually the genes of choice for molecular taxonomy of parasites.

In theory, a variety of nuclear, mitochondrial, and plastid ribosomal sequences are potential amplification targets. These accessory targets reduce the efficiency of amplification of parasite nuclear ribosomal fragments using the Feldman primers. Under certain conditions, one might mistake amplification products of the host's ribosomal sequences for that of an intracellular parasite, giving rise to false positives. In our case, however, we would have to account for 100% of the 'Amakihi sample giving false negatives. We find this suggestion unlikely. Finally, if we subtract out a false positive rate as high (19%) as that suggested to plague certain early PCR tests in humans, we still have significantly higher infection levels (50–19 = 31%) than the previously reported 4% for alien species in Hawaiian lowland forests (van Riper et al. 1982) using the smear method.

Nested primer design, correct magnesium titration for different instruments with different cycling parameters, and high annealing temperatures help to ensure that PCR amplification is accurate and specific. We suggest that it may be necessary to sequence amplification products in each new species tested to verify their source, and that direct sequencing of amplification products is the only accurate way to study the individual selection of parasite genomes within different species of bird host cells over the course of an infection. This precaution should be taken with both the18s rRNA amplification products produced by the reactions we use and the additional TRAP gene test under development (Jarvi et al. *this volume*).