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## SEROLOGICAL RESPONSES AND IMMUNITY TO SUPERINFECTION WITH AVIAN MALARIA IN EXPERIMENTALLY-INFECTED HAWAII AMAKIHI

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**ABSTRACT:** Six of seven Hawaii Amakihi (*Hemignathus virens*) with chronic malarial infections had no increases in peripheral parasitemia, declines in food consumption, or loss of body weight when rechallenged with the homologous isolate of *Plasmodium relictum* 61 to 62 days after initial infection. Five uninfected control amakihi exposed at the same time to infective mosquito bites developed acute infections with high parasitemias. Reductions in food consumption and loss of body weight occurred in all control birds and three of these individuals eventually died. When surviving birds were rechallenged >2 yr later with either the same parasite isolate or an isolate of *P. relictum* collected on the island of Kauai, all individuals were immune to superinfection. Chronically infected birds developed antibodies to a common suite of malarial antigens ranging in size from 22 to 170 kDa that were detectable as early as 8 days post infection on immunoblots of SDS-polyacrylamide gels. Antibodies to this suite of malarial antigens persisted as long as 1,248 days after initial infection and were consistently detectable at times when parasites were not easily found by microscopy on Giemsa-stained blood smears. The immunoblotting method that is described here appears to be an effective technique for identifying birds with chronic, low-intensity malarial infections when circulating parasites are not easily detectable by microscopy. Hawaiian honeycreepers that are capable of recovering from acute infections develop concomitant immunity to superinfection, making them functionally immune in areas where malaria transmission has become endemic.

**Key words:** Concomitant immunity, Drepanidinae, Hawaii Amakihi, *Hemignathus virens*, immunoblot, honeycreeper, premunition, *Plasmodium relictum*.

### INTRODUCTION

High susceptibility of native Hawaiian honeycreepers (Drepanidinae) to introduced avian malaria (*Plasmodium relictum*) is believed to be one of the primary factors responsible for the disappearance of these birds from lowland habitats on the main Hawaiian islands. Van Riper et al. (1986) and C. T. Atkinson (unpubl. data) have shown through detailed field studies that the disease is maintained in mid-elevation forests between 1,000 and 1,500 m where populations of highly susceptible native birds overlap with the mosquito vector, *Culex quinquefasciatus*. At these elevations, the primary reservoirs of infection are native forest birds themselves, with prevalences of infection as high as 40% based on microscopic examination of Giemsa-stained blood smears (van Riper et al., 1986; C. T. Atkinson, unpubl. data).

Iiwi (*Vestiaria coccinea*), Hawaii Amakihi (*Hemignathus virens*), and Apapane (*Himatione sanguinea*) that have been exposed to single infective mosquito bites undergo severe, acute malarial infections that cause declines in food consumption, loss of body fat and weight, and decreases in activity levels that leave acutely ill individuals moribund and highly susceptible to environmental stress and predation by introduced cats, rats, and mongooses (Atkinson et al., 1995, 2000; Yorinks and Atkinson, 2000). Under experimental conditions, mortality ranges from 63% in Apapane to as high as 90% in Iiwi (Yorinks and Atkinson, 2000; Atkinson et al., 1995). Individuals of both species that are able to recover from acute infections develop chronic infections that are not easily detectable by blood smears and concomitant immunity to reinfection with homologous

isolates of the parasite. This acquired immunity would likely allow survivors to persist at elevations where disease transmission has become endemic, possibly giving them a competitive edge over non-immune honeycreepers that have emigrated from higher elevation habitats.

Accurate identification of these chronically-infected survivors is particularly important because they may carry genes responsible for disease resistance, making them good candidates for efforts to restore low elevation native bird populations through either captive propagation or translocation of wild individuals from one site to another. In this study, we document serological responses and immunity to superinfection with malaria in surviving Hawaii Amakihi from a previous experimental study (Atkinson et al., 2000) and demonstrate that a modification of standard immunoblotting methods is an effective serological technique for identifying chronically-infected honeycreepers with subpatent infections.

## MATERIALS AND METHODS

### Rechallenge of survivors

Seven adult Hawaii Amakihi that had recovered from sporozoite-induced experimental malarial infections (Atkinson et al., 2000) and five uninfected amakihi were maintained in captivity in a screened, mosquito-proof aviary for 3 mo prior to this experiment. Birds had been captured in September 1993, in xeric, high elevation habitat (2,000 m) on Mauna Kea (Hawaii, USA). All amakihi were housed individually in 60 × 30 × 30 cm cages and maintained *ad libitum* on a diet of Nectar Plus (Nectar Corporation, Clearwater, Florida, USA) and slices of fresh oranges (Atkinson et al., 2000).

The seven chronically-infected amakihi were re-challenged with three to five infective mosquito bites at either day 61 or 62 post-infection (PI) using colonized *Culex quinquefasciatus* and previously described methods (Atkinson et al., 2000). Five uninfected amakihi were exposed to single infective mosquito bites at the same time to serve as positive controls. Mosquitoes were infected with the same Apapane isolate of *P. relictum* (KV-115) from the island of Hawaii that was used in the initial experimental infections. The isolate was passaged

twice more before being used to infect mosquitoes for the rechallenge experiment described here—once through an experimentally infected amakihi and once through a canary.

All amakihi were weighed and bled via the brachial vein for preparation of thin blood films every 4 days (Atkinson et al., 2000). Thin blood smears were fixed with methanol and stained with 0.01 M phosphate-buffered Giemsa (pH 7.0). Approximately 100  $\mu$ l of whole blood were collected via jugular venipuncture into a heparinized 28 gauge insulin syringe at days 8, 22, and 44 PI from two positive control birds and days 42 and 83 PI from six rechallenged amakihi. Heparinized whole blood was spun in microhematocrit tubes and plasma was separated from packed erythrocytes and frozen for later immunoblot analysis.

At day 106 PI, surviving birds were moved into a screened, mosquito-proof flight cage for long-term maintenance and provided artificial nectar, orange slices and fruit fly pupae *ad libitum*. Birds were captured with a small mist net at 1 to 2 mo intervals and bled via the jugular vein with a heparinized 28 gauge insulin syringe for collection of plasma and preparation of blood smears.

Parasitemia was quantified for the first 106 days of infection by counting the number of infected erythrocytes per 1,000 erythrocytes as described by Godfrey et al. (1987). When parasitemias dropped to extremely low levels as infections became chronic, the average number of erythrocytes per 400 $\times$  microscope field was estimated by calculating average erythrocyte density in five randomly selected microscope fields. Parasitemia was calculated by counting number of infected erythrocytes per 100 400 $\times$  fields and then calculating the approximate number of erythrocytes that were scanned in the 100 fields. Mean ( $\pm$ SD) number of erythrocytes examined per slide using this procedure was 55,700  $\pm$  10,400 ( $n = 158$ ). These parasitemias also were expressed as number of infected erythrocytes per 1,000 erythrocytes. Nectar consumption was recorded daily between 0700 and 0900 for each bird through day 92 PI.

### Strain variation and duration of immunity

Two of five control birds that recovered from acute infection with the KV-115 isolate of *P. relictum* were rechallenged with a single infective mosquito bite at Day 816 PI and six infective mosquito bites at Day 843 PI. Both rechallenges were with the same KV-115 isolate of *P. relictum* that had been stored frozen in liquid nitrogen prior to thawing, deglycerolization, and inoculation into 1-day-old Pekin

ducklings. The isolate was passaged six times in ducklings prior to being used to infect mosquitoes for the rechallenge at Day 816 PI and 10 times prior to the second rechallenge at Day 843 PI. To confirm that infected mosquitoes were transmitting parasites, an uninfected amakihi was exposed to a single infective mosquito bite at the rechallenge at Day 816 and also at Day 843.

Two of the seven original surviving amakihi were rechallenged with a single infective mosquito bite at Day 869 PI and again with six infective mosquito bites at Day 904 PI with an isolate of the parasite (K-1) that originated from pooled blood samples from three wild Elepaio (*Chasiempis sandwichensis*) captured in the Alakai Wilderness Preserve (Kauai Island, Hawaii, USA; 22°9'N, 159°37'W; elevation 1,125 m) in August 1994. This location is five islands and 540 km northwest of where the KV-115 isolate of *P. relictum* was obtained. The K-1 Elepaio isolate was initially isolated in a canary that had been inoculated IV with pooled blood samples and passaged once more in a different canary before being aliquoted, cryoprotected with glycerin, and frozen in liquid nitrogen. Prior to use in this experiment, a frozen aliquot of infected canary blood was thawed, deglycerolized and inoculated into a 1-day-old Pekin duckling. The isolate was passaged nine times in ducklings before being used to infect mosquitoes for the rechallenge at Day 869 PI and 16 times prior to the rechallenge at Day 904 PI. To confirm that infected mosquitoes were transmitting parasites, an uninfected amakihi was exposed to a single infective mosquito bite at the rechallenge on Day 869 PI and also at Day 904 PI.

Following each rechallenge, amakihi were bled from the brachial vein at 4 day intervals for preparation of thin blood smears for 60 days after the rechallenges at Days 816 and 869 PI. Parasitemia was quantified by counting the number of infected erythrocytes per 1,000 erythrocytes (Godfrey et al., 1987).

#### Immunoblot analysis

Banked plasma samples from chronically-infected amakihi were analyzed using a modification of the immunoblot procedure described by Atkinson et al. (1995). In brief, a crude red blood cell extract of *P. relictum* was prepared by infecting 1-day-old Pekin ducklings with thawed, deglycerolized aliquots of either the KV-115 or K-1 isolates of *P. relictum*. When parasitemias reached 10 to 20%, ducklings were exsanguinated. Blood cells were pelleted and resuspended in phosphate-buffered saline (PBS) (pH 7.4) containing 0.15% saponin. The

cells were allowed to lyse and erythrocyte ghosts and intact malarial parasites were washed three times by centrifugation with ice-cold PBS, aliquoted and stored frozen. Immediately before use, a mixture of malarial antigen originating from the KV-115 and K-1 isolates was extracted in 2% sodium dodecyl sulfate (SDS) in 0.1 M tris buffer (pH 6.8), sonicated to shear DNA molecules, and centrifuged at 10,000 g for 10 min. The supernatant was saved for spectrophotometric determination of protein concentration (BioRad DC protein assay kit, Hercules, California, USA) and immunoblot analysis. An aliquot of SDS-extracted malarial antigen was mixed 1:2 with 2% SDS reducing buffer and heated for 4 min at 95 C. A sample containing 400 µg of total protein was loaded onto either a 6%–15% polyacrylamide gradient gel with a 4% stacking gel or a 12% polyacrylamide gel with a 4% stacking gel and separated for 1 hr at 175 V, equilibrated in transfer buffer, and transferred at 100 V for 1 hr to a 0.45 µm polyvinylidene difluoride (PVDF) membrane. Broad range biotinylated molecular weight markers (BioRad) were mixed with the sample, run on each gel and transferred to the PVDF membrane at the same time. Membranes were washed three times in distilled water, dried and stored at –20 C until use.

Before labeling with amakihi plasma, membranes were rewetted with 100% methanol, rinsed in 0.5 M NaCl in 0.01 M tris buffer, pH 7.4 (TBS), blocked for 15 min in 5% non-fat dry milk-0.5 M NaCl-0.01% Tween 20 in 0.01 M tris buffer, pH 7.4 (Saline-Milk-Tween) and placed into a BioRad multiscreen apparatus (BioRad) that divided the membrane into 20 adjacent lanes. Plasma from experimentally infected amakihi was diluted 1:200 with Saline-Milk-Tween, loaded into adjacent lanes, and incubated for 2 h at 37 C. Lanes were washed three times with Saline-Milk-Tween and incubated with rabbit anti-forest bird immunoglobulin secondary antibody diluted 1:4000 in Saline-Milk-Tween for 1.5 hr at 37 C. The secondary antibody had been produced as described previously to the immunoglobulin fraction of five species of native Hawaiian forest birds (Atkinson et al., 1995). Lanes were washed three times with Saline-Milk-Tween and incubated for 1 hr with affinity purified alkaline-phosphatase-labelled goat anti-rabbit antibody or with avidin alkaline phosphatase-labelled conjugate for detection of molecular weight markers (BioRad). Lanes were washed three times with Saline-Milk-Tween, once with TBS, and then developed with an alkaline phosphatase conjugate substrate kit (BioRad). Controls included lanes that were incubated

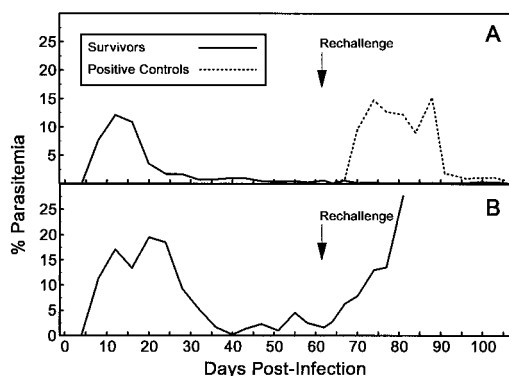


FIGURE 1. Percent parasitemia in surviving amakihi and positive control birds. Surviving amakihi (Survivors) were exposed to a single infective mosquito bite at Day 0 and rechallenged with three to five infective mosquito bites at days 61 or 62 PI. Positive control birds were exposed to single infective mosquito bites at day 62. Data from the first 60 days PI were reported previously (Atkinson et al., 2000). A. Mean parasitemia for six surviving amakihi and five control birds. B. Parasitemia for the single amakihi that survived initial infection with *P. relictum*, but died 19 days after rechallenge with five infective mosquito bites.

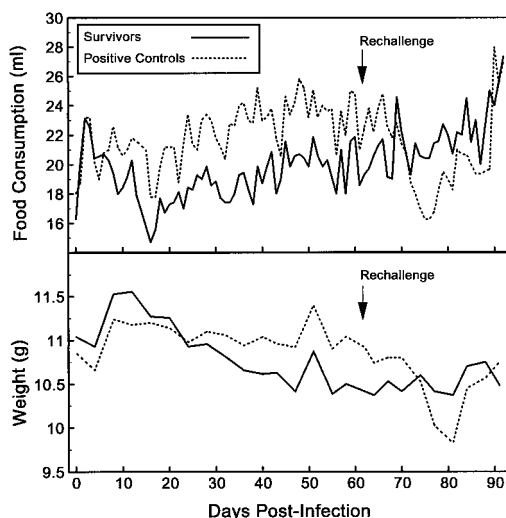


FIGURE 2. Mean daily nectar consumption (ml) and mean weights (g) for rechallenged survivors ( $n = 7$ ) and infected control birds ( $n = 5$ ). Surviving amakihi were exposed to a single infective mosquito bite at Day 0 and rechallenged with three to five infective mosquito bites at days 61 or 62 PI. Uninfected control birds were exposed to single infective mosquito bites at day 62. Data from the first 60 days PI were reported previously (Atkinson et al., 2000).

without primary antibody. Molecular weight estimations of antigens recognized by immune plasma were verified from blots of proteins that were separated on gradient gels.

Birds that died during the course of the experiment were necropsied within 24 hr of death. Representative pieces of all major organs were fixed in 10% buffered formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin.

## RESULTS

### Parasitemia and mortality

Of seven chronically infected amakihi that had recovered from prior infection with *P. relictum*, only one bird exhibited increases in parasitemia after rechallenge at day 62 PI with five infective mosquito bites (Fig. 1). Parasitemia in this individual increased steadily until its death at day 81 PI, reaching a peak of 28% (Fig. 1B). The bird was emaciated at necropsy and had the characteristic gross lesions of acute malaria, including an enlarged and blackened liver and spleen and thin and watery heart blood. The most notable microscopic lesions included deposition of malarial pig-

ment in Kupffer cells of the liver and macrophages of the spleen, mild, multifocal extramedullary erythropoiesis in sinusoids of the liver, and a moderate to marked interstitial pneumonia associated with increases in numbers of granulocytes in the pulmonary interstitium. None of the remaining six rechallenged amakihi exhibited increases in parasitemia or declines in food consumption or body weight (Figs. 1, 2).

All five control amakihi that were exposed to single infective mosquito bites at the same time surviving birds were rechallenged developed acute malarial infections (Fig. 1A). Three of the five control birds died at days 9, 17, and 28 PI with parasitemias of 15%, 24%, and 44%, respectively. All three birds had gross and microscopic lesions that were characteristic of acute, fatal malaria. The two control birds that survived acute infection reached peak parasitemias of 7% and 23% at days 8 and 12 PI, respectively, and subsequent-

ly recovered. Food consumption and weights for control birds declined during the first 16 days of infection, but increased and returned to levels comparable to the rechallenged amakihi as the two surviving control birds recovered from acute infections (Fig. 2).

When four chronically infected amakihi that were still alive at 2 yr PI were re-challenged with multiple infective mosquito bites of either the Kauai (two birds) or Hawaii island (two birds) isolates of *P. relictum*, no increases in parasitemia were observed. Four uninfected amakihi that were exposed at the same time to single infective mosquito bites with either the Kauai (two birds) or Hawaii island (two birds) isolates of the parasite experienced acute infections. Three of these four birds subsequently died from acute malaria at days 13, 17 and 22 PI with parasitemias of 44%, 56% and 63%, respectively.

#### Serological responses

Immunoblot analysis of sequential plasma samples was conducted for six rechallenged and three control amakihi that survived more than 100 days after experimental infection with *P. relictum*. Plasma samples were collected at times ranging from 8 to 1,248 days PI, with most samples collected after birds had recovered from acute infections.

All nine chronically-infected amakihi produced antibodies that recognized a suite of common malarial antigens on immunoblots of SDS-polyacrylamide gels (Fig. 3). Major antigens of approximate molecular weights of 22, 34, 58, 66, 97, 112 and 170 kDa were evident on blots of immune plasma. Depending on reducing conditions, the 170 kDa band often ran as a doublet on SDS-polyacrylamide gels. Bands most commonly recognized by immune plasma from the nine experimentally infected birds were the 34 and 170 kDa antigens (89%), followed by the 58 (78%), 66 (67%), 22 (67%), 112 (56%), 97 (56%), 52 (11%) and 44 (11%) kDa antigens (Fig. 3).

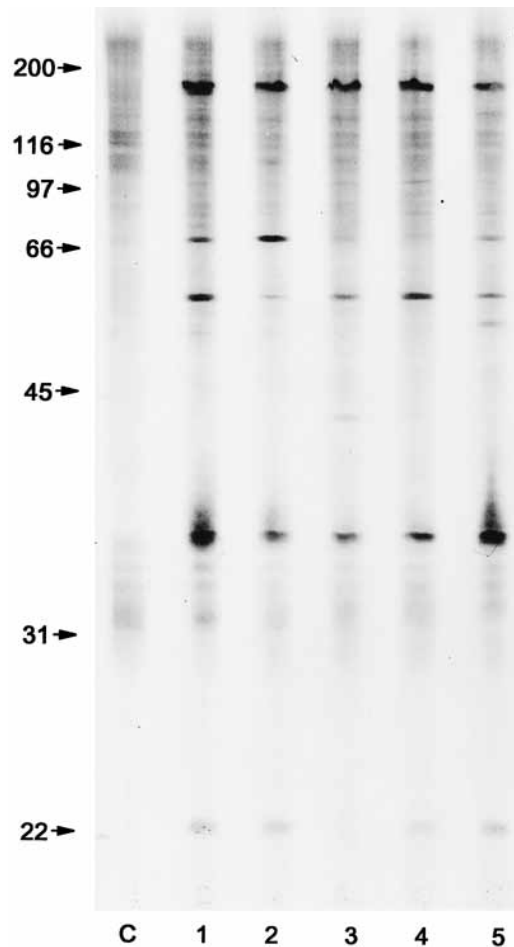


FIGURE 3. Immunoblot analysis of plasma collected from five chronically infected Hawaii Amakihi. Antigens were separated on a 12% polyacrylamide gel with a 4% stacking gel. Lane C (negative control) was incubated with rabbit anti-forest bird Ig, alkaline phosphatase labeled goat anti-rabbit IgG and enzymatic substrates alone. Lanes 1–5 were incubated with plasma collected at days 1,245, 933, 368, 1,027, and 1,248 PI, respectively, from five different amakihi. Erythrocytic parasites were not detected on Giemsa-stained blood smears for samples analyzed on lanes 1–4. Parasitemia in the sample analyzed on lane 5 was estimated to be 0.022 parasitized erythrocyte/1,000 erythrocytes. Molecular weight markers (kDa) are indicated by arrows.

When plasma was analyzed by sequential dates, experimentally infected birds had detectable antibody to the 34 kDa antigen as early as Day 8 PI (Fig. 4), but intensity of labeling was low. The 34, 58, and 170 kDa antigens were usually the

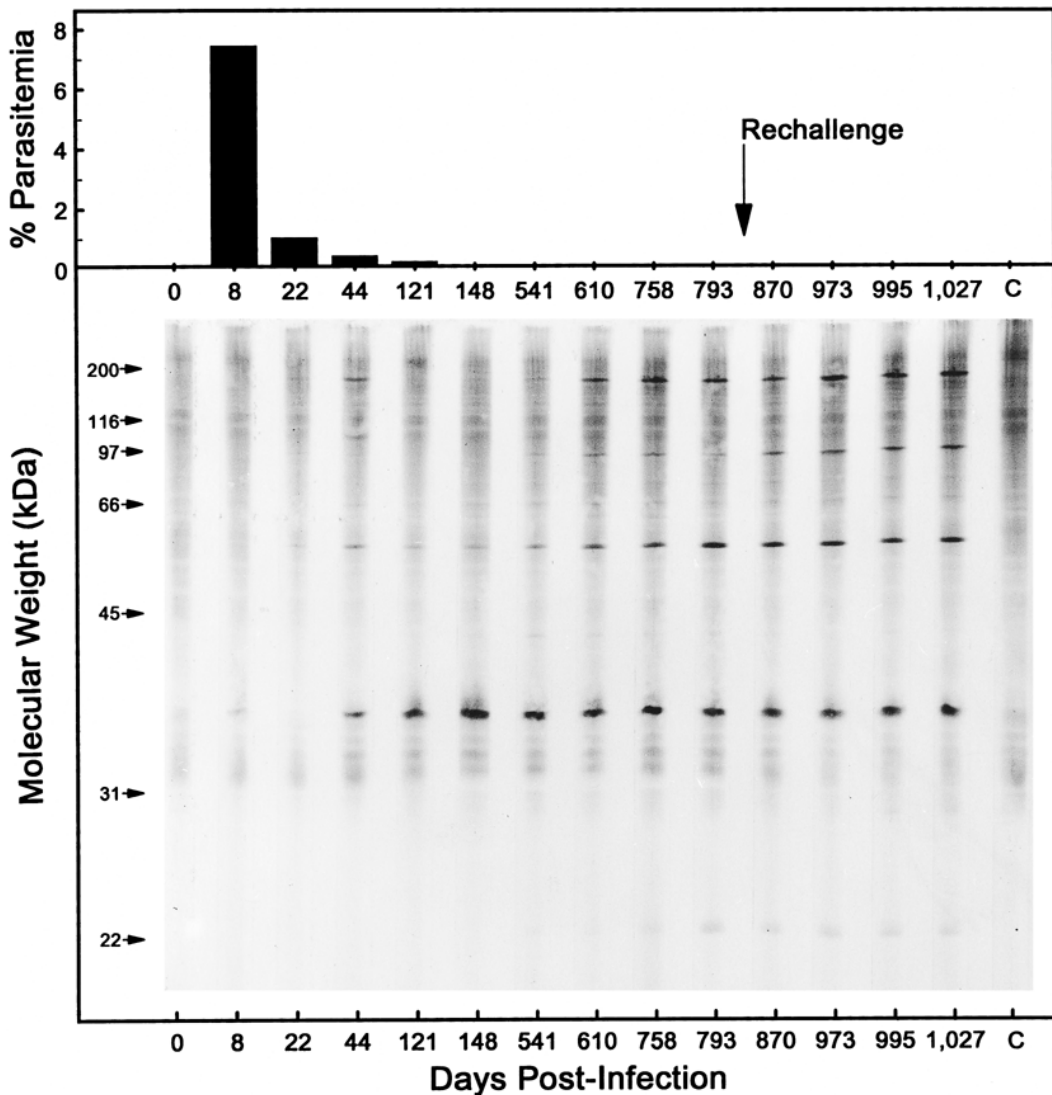


FIGURE 4. Immunoblot analysis and parasitemia in sequential blood samples from a positive control amakihi that was rechallenged at days 816 and 843 PI with a homologous isolate of the parasite. Antigens were separated on a 12% polyacrylamide gel with a 4% stacking gel. Lane 1 (Day 0) was incubated with pre-infection plasma. Remaining lanes were incubated with plasma samples collected at various days post-infection. Lane C (negative control) was incubated with rabbit anti-forest bird Ig, alkaline phosphatase labeled goat anti-rabbit IgG and enzymatic substrates alone. Molecular weight markers (kDa) are indicated by arrows.

earliest ones to be recognized by experimentally-infected birds, but a consistent banding pattern was not usually evident until after day 40 PI (Fig. 4). As these chronic infections progressed, intensity of labeling generally increased, particularly for the 34 and 170 kDa antigens. Detectable antibodies to 22, 66, 97, and 112 kDa malarial antigens did not develop until lat-

er in infections. This was particularly true for the 22 kDa antigen which was not clearly visible on blots before Day 180 PI.

Antibodies to this common suite of malarial antigens persisted in chronically infected amakihi and were evident as long as 1,248 days after initial infection with the parasite. Parasitemias at these dates were less than 0.5% in all infected birds and

could often not be detected on Giemsa-stained blood films. Changes in banding patterns or intensity of labeling were not evident among plasma samples for the four amakihi that were rechallenged between days 816 and 904 PI with either the Kauai or Hawaii isolates of the parasite.

#### DISCUSSION

Six of seven Hawaii Amakihi with chronic malarial infections had no increases in peripheral parasitemia or declines in food consumption or weight when rechallenged by mosquito bite with a homologous isolate of the parasite. This phenomenon, termed concomitant immunity or premunition, was first described >70 yr ago with experimentally-infected canaries (Taliaferro and Taliaferro, 1929), and is based on persistence of a chronic infection that stimulates humoral and cell-mediated immunity to superinfection with homologous isolates of the parasite. These chronic infections can persist for as long as 8 years in infected birds, and probably last for the lifetime of infected individuals (Bishop et al., 1938). We had similar findings and were able to demonstrate that highly susceptible Hawaii Amakihi can remain infected with *P. relictum* for years if they are able to recover from acute infection. These individuals may, however, be vulnerable to relapses and/or superinfection if a compromise in immune function occurs (Norris et al., 1994; Nordling et al., 1998), possibly explaining why one of the seven rechallenged amakihi developed a fatal acute infection after exposure to multiple infective mosquito bites.

We failed to observe increases in parasitemia or changes in banding patterns in the serological analysis of the four amakihi that we rechallenged with Kauai or Hawaii isolates of *P. relictum* >2 yr after initial infection. This suggests that the isolates are antigenically similar and possibly represent identical strains of the parasite (Redmond, 1939; Manwell and Goldstein, 1939; Wolfson and Causey, 1939). These results were somewhat surprising given

the geographic distance between where the isolates were collected, but might be expected if only a few introductions of the parasite have been made to the islands.

While it is clear that acute infections have significant effects on host morbidity and mortality, the effect of chronic malaria on survivorship and fitness of passerine birds has not been well documented. Norris et al. (1994) and Nordling et al. (1998) have recently shown that there is a physiological trade-off between stress associated with reproduction and immune function, leading to decreased ability to mount specific immune responses and increases in the intensity of chronic hemosporidian infections during the breeding season. This trade-off appears to be mediated in part by environmental conditions and availability of food resources (Wiehn and Korpi-mäki, 1998). Unfortunately, key data directly linking chronic hematozoan infection with reduced host fitness in wild populations are limited (Norris et al., 1994; Allander and Bennett, 1995) and virtually nothing is known about the impact of these infections on reproductive success of native Hawaiian forest birds. We have recaptured chronically-infected wild Apapane that are in good body condition with abundant subcutaneous fat over time intervals as long as almost 6 yr at a location in Hawaii Volcanoes National Park where malaria transmission is seasonal (C. T. Atkinson, unpubl. data). We suspect that these birds may, in fact, be more immunologically fit than their uninfected counterparts because they have been "tested" for parasite resistance (Davidar and Morton, 1993).

Field studies of avian species of *Plasmodium* and other closely related hemosporidian blood parasites have been limited by the difficulty in obtaining accurate diagnoses of chronic infections. Parasitemias in these hosts may be so low that they are not easily detectable by conventional microscopy, particularly if the diagnosis is based on examination of a single blood smear collected at a single point in



time. We provided evidence that a modification of standard immunoblotting techniques is a sensitive and specific method for identifying birds with chronic malarial infections. Immune plasma from chronically infected amakihi identified a common suite of malarial antigens ranging in size from 22 to 170 kDa. The antibody response to these antigens was evident as early as 8 days PI when we used a rabbit anti-forest bird immunoglobulin secondary antibody, but was not clearly detectable until approximately 40 days after infection. Development of more specific reagents that can distinguish forest bird IgM from IgG may help improve the sensitivity of the test during early stages of infection. By combining immunoblot analysis of plasma with microscopy, infections can be accurately diagnosed from the time birds first become patent, between 4–8 days after inoculation of infective sporozoites, through chronic stages of infection when numbers of erythrocytic parasites drop to low levels or disappear completely from the peripheral circulation. When applied to epidemiological studies, this technique is a potentially powerful tool for mapping prevalence of malaria in remote forest bird habitats during seasons when transmission and numbers of acute infections are low and where long-term studies are not logistically practical.

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