EXPERIMENTAL INFECTION OF HAWAI`I `AMAKIHI (HEMIGNATHUS VIRENS) WITH WEST NILE VIRUS AND COMPETENCE OF A CO-OCCURRING VECTOR, CULEX QUINQUEFASCIATUS: POTENTIAL IMPACTS ON ENDEMIC HAWAIIAN AVIFAUNA

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EXPERIMENTAL INFECTION OF HAWAI`I `AMAKIHI (*HEMIGNATHUS VIRENS*) WITH WEST NILE VIRUS AND COMPETENCE OF A CO-OCCURRING VECTOR, *CULEX QUINQUEFASCIATUS*: POTENTIAL IMPACTS ON ENDEMIC HAWAIIAN AVIFAUNA

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ABSTRACT: Introduced mosquito-borne avian disease is a major limiting factor in the recovery and restoration of native Hawaiian forest birds. Annual epizootics of avian pox (*Avipoxvirus*) and avian malaria (*Plasmodium relictum*) likely led to the extinction of some species and continue to impact populations of susceptible Hawaiian honeycreepers (Drepanidinae). The introduction of a novel pathogen, such as West Nile virus (WNV), could result in further population declines and extinctions. During September and October 2004, we infected Hawai`i `Amakihi (*Hemignathus virens*) with a North American isolate of WNV by needle inoculation and mosquito bite to observe susceptibility, mortality, and illness in this endemic passerine, and to determine the vector competence of the co-occurring, introduced mosquito *Culex quinquefasciatus*. All experimentally infected Hawai`i `Amakihi became viremic, with a mean titer $>10^5$ plaque-forming units (PFU)/ml, and they experienced clinical signs ranging from anorexia and lethargy to ataxia. The fatality rate among needle-inoculated Hawai`i `Amakihi ($n=16$) was 31.3%, but mortality in free-ranging birds is likely to increase due to predation, starvation, thermal stress, and concomitant infections of avian malaria and pox. Surviving Hawai`i `Amakihi seem to clear WNV from the peripheral blood by 7–10 days postinfection (DPI), and neutralizing antibodies were detected from 9 to 46 DPI. In transmission trials, Hawaiian *Cx. quinquefasciatus* proved to be a competent vector and Hawai`i `Amakihi an adequate amplification host of WNV, suggesting that epizootic WNV could readily become an additional limiting factor of some native Hawaiian bird populations.

Key words: *Culex quinquefasciatus*, experimental infection, Hawai`i `Amakihi, Hawaiian avifauna, vector competence, West Nile virus.

INTRODUCTION

Due to the extreme geographic isolation of the Hawaiian Islands and the absence of many arthropod vectors, island endemic birds evolved in the absence of arboviruses and blood parasites common to mainland birds (van Riper and van Riper, 1985; Torchin et al., 2003). Past and recent serologic studies of introduced mosquitoes, wild birds, domestic fowl, horses, and humans in the Hawaiian Islands have yet to detect avian arboviruses commonly found elsewhere in Pacific and U.S. mainland (Wallace et al., 1964). However, two introduced mosquito-borne avian pathogens, avian pox virus (*Avipoxvirus*; family *Poxviridae*) and avian malaria (*Plasmodium relictum*), are widespread in the islands and are responsible, in part, for the extinction and decline of native Hawaiian birds (Warner, 1968; van Riper et al., 1986; Atkinson et al., 1995). Endemic Hawaiian honeycreepers (Drepanidinae) are particularly susceptible to avian pox and avian malaria (Warner, 1968; Atkinson et al., 1995, 2000), although recent evidence suggests that low-elevation populations of the Hawai`i `Amakihi (*Hemignathus virens*) may be evolving greater immunologic capacity to control malarial infection (Woodworth et al., 2005). Continued introduction of novel pathogens, such as West Nile virus (WNV; family *Flaviviridae*), could potentially have an equally devastating impact on Hawaiian avifauna.

West Nile virus emerged during sum-

mer 1999 in New York state and quickly spread across the North American continent. The virus causes noteworthy morbidity and mortality in a wide range of avian species and is now considered enzootic in North America (McLean et al., 2001; Marra et al., 2004; Kilpatrick et al., 2007). Several *Culex* species have been implicated as enzootic vectors of WNV (Turell et al., 2001, 2005; Goddard et al., 2002). Both local (McLean, 2006) and regional (Koenig et al., 2007; LaDeau et al., 2007) declines in North American avian species have been detected, with sharp declines in some populations of American Crows (*Corvus brachyrhynchos*; Yaremych et al., 2004; Caffrey et al., 2005).

Because WNV is established in California (Reisen et al., 2006b), the Hawaiian Islands are vulnerable to its introduction (Kilpatrick et al., 2004). There is a particular concern that WNV may have significant impacts on the endemic and indigenous avifauna of Hawai`i and other isolated island groups in the Pacific Basin. Island-wide distribution of amplifying host species (e.g., House Sparrow [*Passer domesticus*] and House Finch [*Carpodacus mexicanus*]; Komar et al., 2003), naïve native bird species, and a likely competent vector species (e.g., southern house mosquito [*Culex quinquefasciatus*]; Turell et al., 2005) creates a favorable environment for the establishment and rapid spread of WNV. Based on the susceptibility of mainland North American corvids (Yaremych et al., 2004) and raptors (Wünschmann et al., 2004, 2005; Joyner et al., 2006) to WNV, the Hawaiian Crow or `Alalā (*Corvus hawaiiensis*) and the Hawaiian Hawk or `Io (*Buteo solitarius*) are endangered species that could be negatively impacted by this pathogen. Perhaps of greater concern is the recovery of many honeycreeper populations that could be further hampered by fitness loss or mortality due to a novel pathogen.

In an effort to better understand the risks that WNV poses to Hawaiian avifauna, the objectives of the present study were to 1) determine the susceptibility of a common Hawaiian endemic passerine, the Hawai`i `Amakihi, to WNV; 2) assess the ability of the Hawai`i `Amakihi to serve as an amplifying host of WNV (Komar et al., 2003); and 3) assess the vector competence of a Hawaiian population of *Cx. quinquefasciatus* to serve as an enzootic vector of WNV.

MATERIALS AND METHODS

Capture and care of experimental hosts

Hawai`i `Amakihi (hereafter `Amakihi) of mixed age and sex were captured with mist nets from a xeric, high-elevation (≥1,800 m above sea level) forest on the SW flank of Mauna Kea, Island of Hawai`i (19°50′0″N, 155°35′88″W) between February and June 2004. Mosquitoes are typically absent from these cooler, drier forests, and the prevalence of avian malaria and pox is <0.5% (van Riper, 1991). Captured birds were weighed, sexed, aged by plumage and morphometrics (Lindsey et al., 1998), and transported to a mosquito-proof aviary at Kilauea Field Station (KFS), Hawai`i Volcanoes National Park. Birds in breeding condition (brood patch or prominent cloacal protuberance) were not collected. At KFS, the `Amakihi were initially housed in a free-flight cage measuring 6×3×3 m under natural light cycles and ambient temperature and humidity and fed a diet of Nectar-Plus® (Necton Corporation, Clearwater Florida, USA), orange slices, fly pupae, and scrambled eggs ad libitum. Approximately 14 days before shipping to USGS biosecurity level 3 (BSL-3) facilities at the National Wildlife Health Center (NWHC) in Madison, Wisconsin, birds were moved to individual cages measuring 60×30×30 cm. A daily treatment with fluconazole (10 mg/kg) was initiated at this time to prevent stress-related aspergillosis. `Amakihi were subsequently shipped by overnight air in individual compartments within a screened transport box to prevent exposure to mosquitoes.

At the NWHC, `Amakihi were held individually in cages measuring 244×122×122 cm under the following environmental conditions: 14:10 (L:D) cycle, 22.9±1.13 C (mean±SD), and 45.4±11.6 % RH (mean±SD). Cages were furnished with multiple perches, including artificial foliage. Birds were fed scrambled eggs, fruit fly pupae, wax moth larvae, corn, and mixed fruits ad libitum, and they were

provided 20 ml of Nectar-Plus twice daily. The unconsumed volume of Nectar-Plus was recorded at each feeding, and subjective observations on the amount of supplemental foods was recorded. One of 27 (4%) `Amakihi died from aspergillosis after transport to the NWHC and before experimentation.

House Sparrows, from the greater Madison, Wisconsin, area (43°7′48″N, 89°19′48″W) were captured in drop-in traps (TroyerV-Top Trap, New Haven Manufacturing, New Haven, Iowa, USA). Sparrows were housed in a separate room, in cages measuring 76×46× 46 cm and kept in groups of five to six birds. They were provided millet sprays, mixed seed, a standard medicated turkey chow (Purina Mills, St. Louis, Missouri, USA), and amprolium-treated water (0.5 ml/l) ad libitum. The medicated chow and amprolium were provided to control potential coccidial infections.

Experimental infections of `Amakihi and House Sparrows

Host susceptibility, illness, and mortality were evaluated by experimental infections conducted in the BSL-3 facilities of NWHC during September and October 2004. Daily treatment with fluconazole was terminated 24–48 hr before exposure to WNV (needle inoculation or mosquito bite). A 0.1-ml blood sample was collected from the jugular vein of all birds before WNV exposure to determine pre-exposure serologic status. House Sparrows served as positive controls in inoculation trials because mortality (0–45%) had been observed in previous experiments (Komar et al., 2003; Hofmeister, unpubl. data).

In inoculation trials (IT-1 and IT-2), House Sparrows and `Amakihi were needle-inoculated with the same virus preparation. Five `Amakihi and five House Sparrows were needle-inoculated in IT-1; 16 `Amakihi and six House Sparrows were needle-inoculated in IT-2. Treatment birds were injected subcutaneously on the medial side of the left thigh with 0.1 ml of solution containing 10^5 plaque-forming units (PFU) of WNV 16399-3 in BA-1 medium (1× Medium 199 with 2 mM L-glutamine, 1× minimal essential medium [MEM] nonessential amino acids, 1× MEM vitamin solution, 0.15% sodium bicarbonate [Invitrogen, Carlsbad, California, USA]) with 20% bovine serum albumin, 100 units/ml penicillin, 100 μg/ml streptomycin, and B 1 μg/ml amphotericin). This WNV isolate (16399-3) was originally collected from an American Crow in New York during the 1999 outbreak, passed twice through Vero cell culture, and stored frozen at −80 C. The dose (10^5 PFU) was at the upper range of virus concentration reported for saliva of WNV-infected *Cx. quinquefasciatus* (Vanlandingham et al., 2004) but less than the mean dose of $10^{5.9}$ PFU reported for *Culex pipiens* probing on a live host (Styer et al., 2007).

We also attempted to infect `Amakihi with WNV by mosquito bite. In the mosquito trial (MT), four, naive `Amakihi were exposed overnight to eight to 10 potentially infectious mosquitoes. These mosquitoes had previously blood fed on infected `Amakihi (IT-1), and then they were held for 21–23 days postinfection (DPI) at 26±1 C (see Blood and oropharyngeal sampling). At the time of blood feeding, the host birds had a mean viremia titer of $10^{5.4\pm0.45}$ PFU/ml.

Three, uninfected `Amakihi served as a procedural control group during the MT. These birds were subject to blood feeding by uninfected mosquitoes and the same daily handling and sampling as experimentally infected birds.

Blood and oropharyngeal sampling

During IT-1, needle-inoculated birds were bled daily by jugular venipuncture (0.07 ml) and orally swabbed (Dacron-tipped applicator) 1–4 DPI. Sparrows were euthanized on 7 DPI, and surviving `Amakihi were bled and swabbed at 10, 17, 23, 28, and 46 DPI. During IT-2, we bled and swabbed `Amakihi on alternate days and held birds in an O_2 chamber for 10 min before and after sampling to reduce handling stress. Birds were sampled in a similar manner during the MT. House Sparrows (IT-2) were also bled on this schedule, but they did not receive O_2 treatment. Birds were sampled on alternate days up to 10 DPI and then weekly at 14, 20, and 28 DPI. Profiles of viremia were constructed for each trial with infected `Amakihi. By sampling two groups of birds on alternate days during IT-2, we were able to follow changes in mean viremia every 24 hr. Control and MT birds were sampled every other day starting at 2 DPI. Daily values represent the geometric mean serum titers of these birds. A serum sample was collected from all surviving birds before euthanasia to assess humoral immune response.

Observations and necropsy

Infected and control `Amakihi were observed during the daily morning and afternoon Nectar-Plus feedings and unusual behavior, clinical signs, and mortality were recorded. Birds that died during the study were immediately swabbed, refrigerated, and necropsied within 1–4 hr. Moribund birds were swabbed,

bled, euthanized by CO_2 inhalation, and necropsied. As with moribund birds, survivors in the experimental trials were swabbed, bled, and necropsied on the day of euthanasia. At necropsy, 0.25–0.5-cm^3 samples of skin, liver, lung, heart, spleen, kidney, and cerebrum were collected in duplicate and placed in 1 ml of BA-1 for culture and 300 µl of RNALater (QIAGEN Valencia, California, USA) for molecular studies. A sample from each tissue also was collected and preserved in 10% buffered formalin for histopathology. From selected fatal and surviving cases of WNV-infected (IT-2 and MT) and control (MT) birds, portions of the following organs were immersed in neutral buffered formalin: brain (cerebrum, cerebellum, optic tectum, and brainstem), liver, breast muscle, heart, kidney, skin, and gonad. Tissues were embedded in paraffin, sectioned at 0.5 µm, and stained with hematoxylin and eosin.

Surviving `Amakihi were euthanized on 10 and 28 DPI during IT-2 and on 20 DPI during MT. One bird (IT-1) was observed out to 46 DPI before euthanasia.

Mosquito rearing and blood-feeding conditions

Egg rafts of *Cx. quinquefasciatus* were collected from a low elevation forest (Bryson's Cinders, Island of Hawai`i; 19°27′1″N, 154°55′48″W; 300 m above sea level) and reared to adults in the KFS insectary at 12:12 (L:D) cycle, 27±1 C, and 60–70% RH. Larval mosquitoes were reared in trays (46×36×12 cm) containing 3 l of tap water at a density of approximately 2,000 larvae per tray, and fed a 50:50 brewer's yeast:lactalbumin powder ad libitum. Adults were housed in a screened cage (46×46×46 cm) and maintained on a 3% sucrose solution. Females were blood fed on captive House Sparrows, egg rafts were collected, and larvae were reared to adults (F2).

Adult mosquitoes (F2) were shipped in 4-liter waxed food containers, sealed within a Styrofoam box, to NWHC by a 2-day air carrier. During shipping, mosquitoes were provided a 3% sucrose solution and kept cool with ice packs. At NWHC, F2 adults were housed in the 4-liter waxed food containers or in screened cages (30×30×30 cm) and maintained on 3% sucrose solution in an environmental chamber at 12:12 (L:D) cycle, 26±1 C, and 60–70% RH.

Initial blood feeding occurred 2–4 days after arrival at NWHC when the mosquitoes were 6–9 days old. Mosquitoes were deprived of sucrose at 24 hr and water at 6 hr before blood feeding. Blood feeding commenced at 5:00 PM–7:00 PM in a darkened isolation room when female mosquitoes were introduced into a cylindrical Lucite® chamber measuring 31×15 cm containing an `Amakihi or House Sparrow held within in a small welded wire cage (10×5×5 cm). The bottom of the feeding chamber was covered with moistened paper towels to increase humidity and absorb droppings. Approximately 12 hr later, blood-fed mosquitoes were transferred to 500-ml waxed food containers and maintained in the environmental chamber as described above.

Vector competence and experimental transmission

Both the disseminated virus rate of mosquitoes blood fed on WNV-infected birds (Turell et al., 2001) and evidence of successful transmission of virus from infected mosquitoes to naïve `Amakihi (MT) were used to assess the vector competence of Hawaiian *Cx. quinquefasciatus*. Mosquitoes were infected by allowing 20 unfed mosquitoes to blood feed overnight on WNV needle-inoculated House Sparrows and `Amakihi at 2–4 DPI (IT-1). Twenty-one to 23 days after blood feeding, mosquitoes were killed by freezing at −80 C and dissected. The legs of individual, potentially infected mosquitoes were removed with fine forceps, frozen at −80 C, and later assayed for virus. To prevent cross contamination between individual mosquito dissections, forceps were sequentially rinsed in 50% bleach, distilled water, and 95% ethanol and then flamed dry. Individual mosquitoes were considered to have a disseminated infection if West Nile viral RNA was detected in detached legs.

Mosquito transmission was evaluated in the MT, by exposing four naïve `Amakihi individually to potentially infective mosquitoes and monitoring for viremia and clinical signs as described above. After blood feeding, mosquitoes were processed for detection of disseminated virus. To evaluate potential vertical transmission by Hawaiian *Cx. quinquefasciatus*, egg rafts were collected from mosquitoes that had previously blood fed on infected `Amakihi and House Sparrows in IT-1. Three days after blood feeding, a small plastic cup containing an infusion of horse manure was placed in the containers for oviposition. Larvae from individual egg rafts were reared to adults at 26 C in 350-ml plastic food containers. Whole bodies of adult female progeny (F1) were individually frozen at −80 C and later assayed for presence of West Nile viral RNA. It should be noted that both the dissemination and filial infection rates are based on detection of West Nile viral RNA and therefore do not

necessarily represent viable, infectious virus (Nasci et al., 2001).

Samples, virus assays, and antibody testing

Sera were collected by adding 70 µl of whole blood to 315 µl of BA-1 media, allowing the diluted blood to clot for 30 min, and then centrifuging the mixture in CapiJect® tubes (Terumo Medical Corporation, Somerset, New Jersey, USA). The final serum dilution was 1:10, assuming a 50:50 ratio of packed cells to serum in whole blood. Oral swabs were added to 1 ml of BA-1 media. Individual mosquitoes, dissected body parts, and whole mosquito pools were placed in dry vials. All samples (serum, oral swabs, and mosquitoes) were held on wet ice until processing and then stored at −80 C, within 1 hr, until testing.

For virus quantification, a 12-well plate, mini-plaquing technique was used to accommodate the small sample volumes. Each well was plated with 1 ml of Vero cells in complete M199 media (2×10^5 cells/ml), and plates were incubated for 4 days at 37 C, 5% CO_2. Serum and swab samples were diluted in M199 media containing 10% fetal bovine serum, added to confluent Vero cell monolayers, and incubated for 45 min before 1 ml of complete overlay was added (Beaty et al., 1995). Serum and swab samples were screened at a 1:10 dilution, and positive samples were titered on a six-well plate in a twofold series (1:20–1:640). Tissue samples in BA-1 media were processed for 1 min in a mini-bead beater instrument (BioSpec Products, Inc., Bartlesville, OK, USA) by using 2.0-mm zirconium beads before being added at a starting dilution of 1:5 to Vero cells cultured on a six-well plate, as described previously. Cultures were checked for plaque formation at 96, 120, and 144 hr postinfection, and virus concentrations were reported as PFU/ml, PFU/swab, or PFU/0.5-cm^3 tissue. Samples with only 1 to 2 PFU (corresponding to a threshold of detection of 50 PFU/ml of serum) were repeated to verify the presence of virus.

Mosquitoes from the F1 generation were screened for infection with WNV by either SYBR® Green (Quantitect SYBR® Green reverse transcriptase-polymerase chain reaction [RT-PCR] kit, QIAGEN) or TaqMan (QuantiTect Probe RT-PCR, QIAGEN) RT-PCR (Papin et al., 2004). For SYBR Green RT-PCR, a 2-µl volume of extracted RNA from individual mosquito bodies or legs was introduced into a 20-µl RT-PCR reaction containing 1 µM each of WNV envelope glycoprotein gene (WNENV)-forward and WNENV-reverse primers (Lanciotti et al., 2000) and reaction components recommended by the manufacturer. Amplification conditions were an initial cycle of 30 min at 50 C for reverse transcription, 15 min at 95 C for initial PCR activation, followed by 40 cycles consisting of a 15-sec denaturation at 94 C, a 30-sec annealing at 50 C, and a 30-sec extension at 72 C. For TaqMan RT-PCR, the same volume of extracted RNA was introduced into a 20-µl RT-PCR reaction containing 1 µM each of WNENV-forward and WNENV-reverse primers, 0.25 µM WNENV-probe (Lanciotti et al., 2000), and reaction components recommended by the manufacturer. Amplification conditions consisted of an initial cycle of 30 min at 50 C for reverse transcription, 15 min at 95 C for initial PCR activation, followed by 40 cycles with a 15-sec denaturation step at 94 C, a 60-sec annealing and extension step at 60 C. All samples were amplified on an ABI Prism® 7000 Detection System (Applied Biosystems, Foster City, California, USA).

SYBR Green RT-PCR reaction products with a cycle threshold (CT) ≤35 and a melt curve analysis consistent with the positive control WNV RNA, and TaqMan RT-PCR results falling between a CT of >30 and ≤35, were further analyzed by conventional one-step RT-PCR (OneStep RT-PCR kit, QIAGEN). Based on analysis of the WNV-E protein coding sequence (GenBank AF196835), primers WNV-E1241s (5′-GAGCGCAGAGACTAGC-3′) and WNV-E1463a (5′-CAACTGCGA-GAAACGTGAG-3′) were selected using Oligo 6 software (Molecular Biology Insights, Inc., Cascade, Colorado, USA) that amplified a 223-base pair (bp) E protein fragment. The 223-bp fragment was amplified in a 25-µl reaction mixture containing 1× OneStep RT-PCR buffer (QIAGEN), 400 µM of each deoxynucleoside triphosphate, 1 µM of primers WNV-E1241s and WNV-E1463a, 1 µl of OneStep RT-PCR Enzyme Mix (QIAGEN), 10 units of Prime RNase inhibitor (Eppendorf, Westbury, New York, USA), and 2.5 µl of RNA extract. Amplification was performed on a thermocycler (MJ Mini Gradient Thermocycler, Bio-Rad, Hercules, California, USA) using an initial cycle of 30 min at 50 C for reverse transcription, 15 min at 95 C for initial PCR activation, followed by 40 cycles consisting of a 30-sec denaturation step at 94 C, a 30-sec annealing step at 55 C, and a 1-min extension step at 72 C. These were followed by a final 4-min extension step at 72 C. The RT-PCR master mix was prepared in a dedicated PCR preparation hood and template RNA was added in a room in which PCR product was not analyzed. A negative control reaction (distilled H_2O) was included in each group of RT-PCR reactions.

Conventional RT-PCR products were analyzed on a 2% agarose gel, photographed under UV illumination, and transferred to nylon membrane (Millipore, Billerica, Massachusetts, USA) by the method of Southern (1975). Immobilized DNA fragments were specifically identified by hybridization at 42 C with a horseradish peroxidase-labeled probe made by RT-PCR using WNV-E1362s and WNV-E1362a 5′-CGGAGGCATGTCCTGGATAAC-3′ (ECL, GE Healthcare, Little Chalfont, Buckinghamshire, England). Hybridization solutions and washes were as recommended in the ECL Handbook (GE Healthcare). Chemiluminescent activity was recorded on Biomax Light film (Eastman Kodak, Rochester, New York, USA) after a 1-min exposure and scanned under white light conditions. The presence of a 223-bp RT-PCR product was regarded as a positive test result (Fig. 1).

Serum samples were also screened on six-well Vero cell plates at a 1:20 dilution in BA-1 for WNV-neutralizing antibodies by plaque reduction neutralization test (PRNT) (Beaty et al., 1995). Serum dilutions were incubated with a test dose of WNV isolate 16399-3 for 1 hr at 37 C. The test dose of virus was diluted to contain between 60 and 80 PFU. A serum sample was considered positive for antibodies to WNV if a 90% plaque reduction ($PRNT_{90}$) was observed.

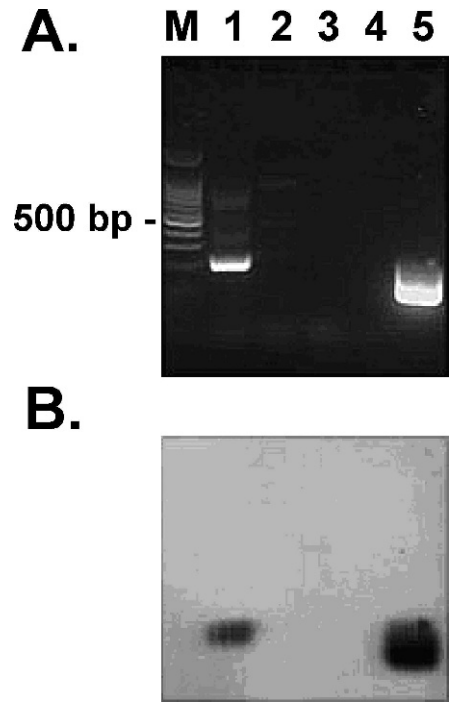

FIGURE 1. Reverse transcriptase-polymerase chain reaction (RT-PCR) amplification of RNA extracted from Hawaiian *Culex quinquefasciatus* legs 10 days after exposure to a West Nile virus (WNV)-infected House Sparrow. (A) RT-PCR products were analyzed on a 2% agarose gel containing Vistra green dye (1:10,000, GE Healthcare). Lane M contained 100-bp ladder (New England Biolabs, Ipswich, Massachusetts, USA). Lane 1, F1 mosquito F1-112 fed upon WNV-infected House Sparrow; lane 2, negative control mosquito legs; lane 3, RNA extraction control distilled H_2O; lane 4, RT-PCR master mix negative control; and lane 5, WNV RNA extracted from infected Vero cells. (B) RT-PCR products from gel in A were transferred to nylon membrane and detected with horseradish peroxidase-labeled probe WNV-E1362s.

Data and statistical analyses

Survivorship was examined with the Kaplan-Meier product limit estimator, and a log-rank test was used to compare survival distributions between needle-inoculated, mosquito-infected, and procedural control birds. For analysis, we combined two `Amakihi from the MT trial with the procedural control group. These two `Amakihi had been exposed to infected mosquitoes but neither developed a detectable viremia, clinical signs, or antibody response, and they were reclassified as uninfected. Repeated measures analyses of variance were used to analyze food consumption (Nectar-Plus), and virus dissemination rates were compared with Fisher exact tests. All statistical analyses were performed with SYSTAT® 10.2 software (Systat Software, Inc., San Jose, California, USA).

RESULTS

Viremia, viral shedding, and antibody response

All needle-inoculated `Amakihi (IT-1 and IT-2; $n=21$) had detectable viremia 1 DPI, and surviving birds remained viremic 4–7 DPI (median=5; $n=13$), with geometric mean titers $>10^5$ PFU/ml blood from 1 to 4 DPI (Fig. 2). Peak viremia titers in needle-inoculated `Amakihi (IT-2; $n=16$) occurred 1–5 DPI (median 2 DPI) and ranged from $10^{6.7}$ to $10^{8.9}$ PFU (geometric mean±SD; $10^{7.8\pm0.6}$). After peak viremia in all needle-inoculated birds, virus titer declined sharply. Mosquito-infected birds ($n=2$) had detectable viremia on 2 DPI (the first day blood was drawn), and they remained viremic until death at 6–8 DPI. Peak viremia titers in mosquito-infected `Ama-

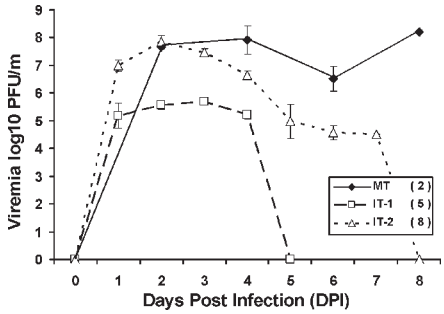

FIGURE 2. Serum viremia profiles for Hawai`i `Amakihi exposed to West Nile virus (geometric mean±SD). Number of individual birds in parentheses.

kihi ranged from $10^{7.6}$ to $10^{8.4}$ PFU (geometric mean±SD; $10^{8.0\pm0.6}$), and geometric mean titers for 6 and 8 DPI were higher than those for needle-inoculated birds at corresponding DPI (Fig. 2). Needle-inoculated and mosquito-infected `Amakihi shed virus orally from 1 to 10 DPI. Virus concentrations from swabs were typically lower than corresponding viremias and ranged from $10^{2.3}$ PFU/swab (10 DPI) to$10^{7}$ PFU/swab (5 DPI). Peak oral shedding in needle-inoculated `Amakihi followed peak viremias and occurred between 4 and 7 DPI (median, 5 DPI), with a geometric mean±SD titer of $10^{5.4\pm0.7}$ PFU/swab. By 7–10 DPI, virus was not detected in surviving birds (Fig. 2). Viremia titers among House Sparrows ranged from $10^{5.3}$ to $10^{7.7}$ PFU between 1 and 2 DPI.

All birds were seronegative for antibodies to WNV by the PRNT assay before experimental infection. We tested final serum samples (collected at time of death or euthanasia) from 18 of the 21 needle-inoculated `Amakihi by PRNT. Sixty-one percent (11/18) tested positive for WNV antibodies. `Amakihi fatalities in IT-1 were seronegative by $PRNT_{90}$ from 4 to 18 DPI. The sole surviving bird in IT-1 had a reciprocal $PRNT_{90}$ of 160 on 28 DPI, >1,280 on 39 DPI, and >640 on 46 DPI. Neutralizing antibodies were detected earlier in the five surviving birds euthanized at 10 DPI during IT-2. Reciprocal $PRNT_{90}$ titers among these birds ranged from 20 to 160. All but one bird ($n=6$) in IT-2 had reciprocal $PRNT_{90}$ titers of 620 when euthanized at 28 DPI. One mosquito-infected bird had seroconverted with a low titer (reciprocal $PRNT_{90}$ of 20) at the time of its death on 10 DPI.

Viral isolation from tissues

Five WNV-infected `Amakihi from MT and IT-2 were selected for tissue viral culture (Table 1). This subsample included birds that died within 10 DPI, were moribund, or survived healthy to 10 DPI. Two control birds euthanized at the end of the study were included for comparison. West Nile virus was detected by tissue culture in all tissues from MT birds that died and generally in excess of 10^{7} PFU/sample. West Nile virus was not uniformly detected in the tissues of two surviving birds from IT-2, and the quantity of virus did not exceed $10^{2.5}$ PFU/sample. In contrast, virus was detected in all tissues

TABLE 1. West Nile virus isolation from various`Amakihi tissues. Virus titer is presented in log 10 plaque-forming units per 0.5 cm^3 of tissue.

			Tissue						
Bird	Trial	DPI	Skin	Liver	Lung	Heart	Spleen	Kidney	Brain
289[a]	MT	8	7.3	7.3	7.3	7.6	6.3	7.8	5.2
290[a]	MT	10	7.5	7.9	7.6	7.3	7.8	7.7	7.3
245	IT-2	9	0	0	0	2.1	0	1.7	1.7
287	IT-2	9	7.1	2.3	7.1	6.5	2.0	7.5	7.3
240	IT-2	10	2.5	0	1.7	2.1	1.9	1.0	0
246	Control	14	0	0	0	0	0	0	0
41590	Control	14	0	0	0	0	0	0	0

[a] Fatal case.

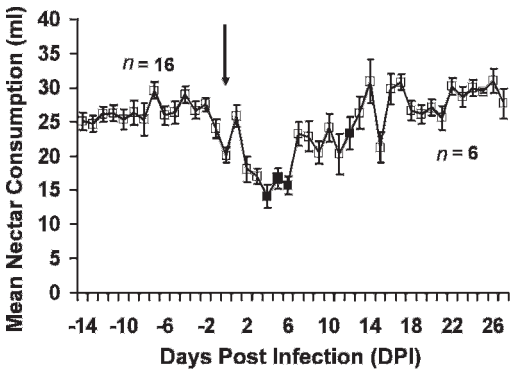

FIGURE 3. Inoculation trial 2 and 3. Mean±SE daily Nectar-Plus consumption (milliliters) pre- and postinfection with West Nile virus (arrow indicates date of inoculation). Filled data points represent mortality event(s).

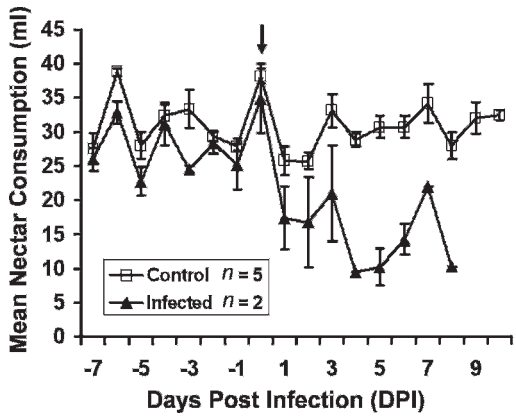

FIGURE 4. Mosquito trial 2. Mean±SE daily Nectar-Plus consumption (milliliters) pre- and postinfection with West Nile Virus (arrow indicates date of inoculation). Control and infected birds were bled and swabbed every other day.

of the third IT-2 bird at the time it was euthanized on 9 DPI. Virus levels were low ($10^{2.0}$–$10^{6.5}$ PFU/sample) in spleen, liver, and heart, but $>10^{7.0}$ PFU/sample in the remaining tissues. This bird seemed healthy before euthanasia. No virus was detected in the tissues of control birds.

Clinical signs, mortality, gross pathology, and histopathology

All infected birds underwent a period of anorexia that coincided with detectable viremia. Anorexia started 1–3 DPI and continued for 4.1±0.81 days (mean±SE; $n=10$). During this time, there was a significant reduction in Nectar-Plus consumption over time (IT-2 and IT-3: $F=24.3$; df=7,98; $P<0.0005$) between the 7 days before and 7 days after inoculation, with the average daily consumption of Nectar-Plus per bird dropping from 27.0±0.73 to 18.7±1.63 ml (geometric mean±SE) (Fig. 3). Similarly, the two infected birds in MT showed a significant change in Nectar-Plus consumption ($F=11.0$; df=7,21; $P<0.0005$) over time. The treatment group-time interaction was also significant ($F=10.03$; df=7,21; $P<0.0005$), indicating a significant difference in Nectar-Plus consumption over time between controls and infected birds (Fig. 4). The average daily consumption of Nectar-Plus in mosquito-infected birds dropped from 27.3±1.39 to 15.9±1.84 ml, whereas consumption in the control group remained unchanged (before, 31.0±1.55 ml; after, 29.9±1.24 ml).

Signs of illness, ranging from general lethargy and ruffled feathers to ataxia and paresis, were present in 55.6% (10/18) of experimentally infected `Amakihi (IT-2 and MT). Legs and wings held abnormally from the body or inability to perch or fly were the most common signs of ataxia and paresis (Table 2). The timing of these clinical signs followed increases in viremia. Approximately half (four of 10) of the individuals with neuromuscular signs survived.

The combined fatality rate for needle-inoculated `Amakihi in the two trials was 43% (nine of 21) and ranged from 80% (four of five) in IT-1 to 31% (five of 16) in IT-2 (Fig. 5). The fatality rate in MT was 100% (two of two) among infected `Amakihi. However, none of the procedural controls ($n=3$) or uninfected `Amakihi ($n=2$) in MT died during the 21-day test period. Based on the Kaplan-Meier analysis, survivorship was significantly different among the three treatment groups ($\chi^2=6.304$, df=2, $P=0.043$). Actual mean survival time±SE for the five `Amakihi succumbing to infection in IT-2

TABLE 2. Survivorship and clinical signs of Hawaii `Amakihi experimentally infected with West Nile virus (WNV).

Outcome[a]	*n*	Anorexia no.(%)	Lethargy no. (%)	Ruffled no. (%)	Ataxia no. (%)		Paresis no. (%)	
					Foot	Wing	Perch	Fly
Died	7	7 (100)	5 (71)	4 (57)	1 (14)	1 (14)	3 (43)	4 (57)
Survived	11	11 (100)	4 (36)	2 (18)	1 (14)	0 (0)	2 (18)	3 (27)
Control[b]	3	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

[a] Outcome of 18 `Amakihi infected with WNV by inoculation (16) or mosquito bite (two).

[b] Uninfected individuals bled and swabbed similarly to infected birds.

was 6.9±1.56 days (median=5.5, range= 4.5–13 days). During IT-2 and MT, most mortality (six of seven) occurred 3–5 days after peak viremia. One bird died 11 days after peak viremia. The fatality rate was lower in the needle-inoculated House Sparrows that served as positive controls. No mortality occurred in five House Sparrows inoculated in IT-1 but 30% (two of six) mortality was observed in inoculated House Sparrows in IT-2. The fatality rate was 18% (two of 11) for the combined House Sparrow trials.

At necropsy, fatal cases exhibited emaciation (11/11), hepatomegaly (four of 11), splenomegaly (seven of 11), calvarial hemorrhage (five of 11), prominent cerebral vessels (one of 11), and prominent dural vessels (one of 11). Gross lesions were not observed in three fatal cases at ≤5 DPI. In healthy survivors, euthanized between 10 and 28 DPI, hepatomegaly (two of 11), splenomegaly (three of 11), calvarial hemorrhage (five of 11), and prominent dural vessels (one of 11) was observed. Enlarged livers and spleens were observed in birds euthanized at 10 DPI. No emaciation (zero of 11) or changes associated with cerebral vessels (zero of 11) was seen in these surviving birds. Gross lesions were not observed in the three control `Amakihi. Changes associated with common opportunistic infections such as aspergillosis and coccidiosis was not observed except for suspected intestinal aspergillosis in one fatal case.

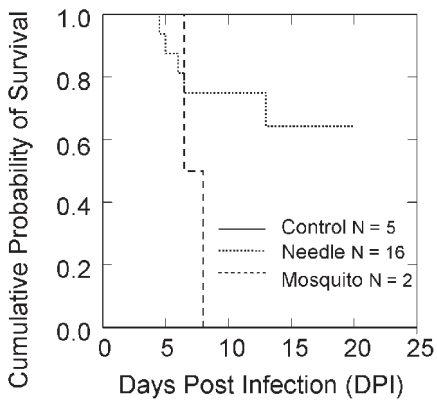

FIGURE 5. Kaplan-Meier survival estimates for West Nile virus-infected Hawai`i `Amakihi. The inoculation trial is based on inoculation trial (IT)-2 results. IT-1 was excluded from the analysis. The control group represents both three procedural control birds and two mosquito trial birds that showed no signs of infection.

In birds with WNV-positive organs, the presence and severity of microscopic lesions generally correlated with the tissue viral load (Table 1). Among the infected birds examined, fatal cases (MT birds 289 and 290) with the highest tissue viral load in multiple tissues also had acute, moderate hepatocyte necrosis, which was accompanied, in one case (bird 290), by acute renal tubular nephrosis with hyaline casts and acute testicular seminiferous tubule necrosis. One surviving individual (IT-2 bird 287), which had several tissues with a high viral load, exhibited mild perivascular infiltrates of mononuclear cells in hepatic portal triads and central veins. Surviving birds (IT-2; Table 1) with relative low tissue concentrations of WNV (virus at or near the low threshold of detection) had either no significant lesions (bird 245) or a

mild, multifocal nonsuppurative myocarditis (bird 240). Lesions were not detected in the tissues of the two control birds (birds 246 and 41590).

Vector competence

Forty-one percent (33/81) of Hawaiian *Cx. quinquefasciatus* that fed on viremic House Sparrows (IT-1) had disseminated infections on 21–23 DPI. The geometric mean±SD viremia for these five birds (R1-24, R22, R25, R9, and R6) 1 day before feeding was $10^{6.4\pm1.1}$ PFU (range= $10^{5.3}$–$10^{7.7}$ PFU). At 21–23 DPI, the disseminated infection rate in Hawaiian *Cx. quinquefasciatus* that fed on viremic `Amakihi (IT-1) was 77% (26/34). The mean±SD viremia for the four host birds over a 3-day feeding period was $10^{5.5\pm0.3}$ PFU (range=$10^5$–$10^6$ PFU). There was a significant difference in dissemination rates between mosquitoes that blood fed upon House Sparrow versus `Amakihi (χ^2=11.0, df=1, P=0.001). During the mosquito transmission trial (MT), one to two mosquitoes with disseminated infections fed on each of three of the four naïve `Amakihi that were exposed. Two of the three (67%) `Amakihi became infected with a detectable viremia.

Nine of 185 (4.9%) individually tested female F_1 progeny were identified as positive for West Nile viral RNA by Taqman RT-PCR or detection of the 223-bp RT-PCR product (Table 3). There were West Nile viral RNA-positive females among the F1 progeny of females that had fed on infected House Sparrows (two of four) and `Amakihi (one of two). Seven of the nine positive progeny originated from a single egg raft from which 37 individuals were tested.

TABLE 3. Summary of West Nile virus vertical transmission by Hawaiian *Culex quinquefasciatus*.

Host[a]	Infection rate[b]	F1 progeny		
		No. positive	No. individuals	Infection rate
HOSP R22	15	1	36	2.8
HOSP R1-24	36	0	3	0
HOSP R25	88	7	37	18.9
HOSP R6	10	0	49	0
(Subtotal)		8	125	6.4
HAAM 251	Unknown	0	40	0
HAAM 201	Unknown	1	20	5
(Subtotal)		1	60	1.67
Total		9	185	4.86

[a] HOSP = House sparrow, *Passer domesticus*; HAAM = Hawai`i `Amakihi, *Hemignathus virens*.

[b] Infection rate of female mosquitoes having blood fed on host.

DISCUSSION

`Amakihi were susceptible to WNV with all needle-inoculated and most mosquito-exposed birds becoming viremic and shedding virus by 1 DPI. Infected birds, representing both surviving and fatal cases experienced clinical signs and gross pathology consistent with WNV infection (Steele et al., 2000; Komar et al., 2003; Weingartl et al., 2004; Gibbs et al., 2005; Clark et al., 2006; Nemeth et al., 2006). These observations along with the high viral loads found in the tissues of fatal cases and presence of histopathologic lesions in some of organs of the same cases indicate that mortality was due to WNV. Given the 49% reduction in fatality rate between IT-1 and IT-2, we conclude that the stress of excessive handling (daily bleeding, swabbing, and mosquito blood feeding) during IT-1 contributed significantly to mortality. A better estimate of the fatality rate for needle-inoculated birds was obtained in IT-2 when handling of experimental birds was reduced to every other day. This more moderate fatality rate is comparable with experimental infections in North American noncorvid passerines such as the Common Grackle (*Quiscalus quiscula*) and House Finch (Komar et al., 2003; Reisen et al., 2005).

Although it is unwise to extend fatality rates derived from laboratory studies directly to field situations, the clinical signs observed in surviving birds during the study suggest that infected `Amakihi

may experience higher mortality in the wild. The general lethargy and neuromuscular impairment experienced by infected birds is likely to increase predation on wild `Amakihi. Paresis, particularly the inability to perch, would effectively ground afflicted birds and expose them to nonnative mammalian predators (Atkinson, 1977). The anorexia observed in all infected birds might also have a profound effect on survivorship in the wild. Anorexic birds could succumb directly to starvation or fail to thermoregulate during cool, nighttime temperatures (Atkinson et al., 2000).

Concomitant infections of avian malaria and pox might also increase mortality among WNV-infected wild birds through synergistic effects. Acute and chronic infections of avian malaria and pox are common in Hawaiian avian communities, with 28–90% of `Amakihi chronically infected with malaria (Woodworth et al., 2005) and 2–10% affected with pox-like lesions (van Riper et al., 2002; Atkinson et al., 2005). Given these prevalences, the frequency of concomitant infections with WNV could be relatively high. The immunosuppression associated with pox in particular (Smith and Kotwal, 2002) could have a profound synergistic effect on WNV mortality. Considering both environmental stressors and concomitant infections, mortality of WNV-infected birds in the wild could exceed the 43% recorded in the present study.

Our data suggests `Amakihi populations might be adversely impacted by WNV but would WNV be efficiently transmitted in Hawaiian forest bird habitat? Transmission requires an efficient amplification host and a competent vector. The titers and duration of viremia in infected `Amakihi were most comparable with House Finches (Komar et al., 2003; Reisen et al., 2005). Because the House Finch has been implicated as a WNV amplification host in North America (Komar et al., 2001, 2003), `Amakihi might be expected to serve a similar role in Hawaiian forests. Nonnative house finches and Japanese White-eyes (*Zosterops japonicus*) also occur in Hawaiian forests, and, based on the observed viremia after experimental infection, they have the potential to serve as efficient amplification hosts as well (Hofmeister, unpubl. data). Our observation that more mosquitoes became infected after feeding on viremic `Amakihi than on House Sparrows is best explained by the shorter duration and lower titer of infectious viremia in House Sparrows compared with `Amakihi and by the experimental timing of mosquito blood feeding.

Although `Amakihi may be a competent amplification host according to criteria set forth by Komar et al. (2001, 2003), others (Reisen et al., 2005, 2006c) have argued that efficient amplification hosts need to experience higher viremias ($>10^7$ PFU/ml)—characteristic of WNV infection in corvids—to compensate for the relatively low vector competence of most North American *Culex* spp. Vector competence for WNV among North American *Culex* species varies considerably, with *Cx. quinquefasciatus* typically ranking among the least efficient laboratory vectors (Sardelis et al., 2001; Goddard et al., 2002; Reisen et al., 2005). However, extensive variation in vector competence for WNV has been reported among geographic populations of *Cx. quinquefasciatus* (Sardelis et al., 2001; Goddard et al., 2002). In California, transmission rates (based on detection of expectorated virus) for *Cx. quinquefasciatus* populations ranged from 0% to 71% (Goddard et al., 2002; Vaidyanathan and Scott, 2007), indicating that some populations are efficient laboratory vectors. We recorded a disseminated infection rate of 74% in Hawaiian *Cx. quinquefasciatus* having blood fed on viremic `Amakihi, and, although methodologic differences prevent us from making a direct comparison, this suggests that Hawaiian *Cx. quinquefasciatus* may be similar in competence to the most

competent *Culex* spp. and *Cx. quinquefasciatus* populations tested to date.

In addition to high dissemination rates in Hawaiian *Cx. quinquefasciatus*, we observed filial infection rates in progeny of females with disseminated infections higher than reported previously in *Cx. quinquefasciatus* from laboratory (Goddard et al., 2003) or field (Reisen et al., 2006b) studies. Although the accuracy of our estimate is limited by our molecular approach (Nasci et al., 2001; Farajollahi et al., 2005) and small sample size, it provides some evidence of vertical transmission and suggests a possible overwintering mechanism in Hawai`i for virus at higher elevations where adult mosquitoes occur seasonally (LaPointe, 2000; Ahumada et al., 2004).

A possible additional route of transmission is suggested by the observed oral shedding of WNV-infected `Amakihi. Komar et al. (2003) demonstrated experimental per os transmission in House Sparrows by administering an aqueous solution containing $10^{7.4}$ PFU of WNV. We detected similar concentrations of virus in oral swabs from infected `Amakihi, and it is conceivable that infected individuals might directly transmit WNV by regurgitating food for nestlings or indirectly transmit to other birds through nectar feeding.

In conclusion, our findings suggest that should WNV be introduced to the Hawaiian Islands, epidemiologic conditions are conducive to the establishment, amplification, and rapid spread of the virus to native forest birds. Although the observed mortality in this study may be insufficient to cause a permanent decline in common honeycreeper populations, the additive effects of environmental stressors and/or synergistic effects of concomitant infections may increase mortality to a level that could be devastating to Hawai`i `Amakihi and other honeycreeper populations. Additionally, WNV could jeopardize recovery of lowland populations of the endangered Hawaiian Hawk and would likely be an insurmountable obstacle to the successful restoration of the critically endangered Hawaiian Crow. Populations of common and recovering endemic species could become restricted to the high elevation forest where diminished rainfall and cool temperatures limit vector abundance and virus replication and dissemination (LaPointe, 2000; Dohm et al., 2002; Reisen et al., 2006a). Additional research to determine the effect of concomitant infections in Hawaiian honeycreepers is essential to gain a full understanding of the potential impact of WNV on Hawaiian avifauna.

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