Efficacy of Three Vaccines in Protecting Western Scrub-Jays (*Aphelocoma californica*) from Experimental Infection with West Nile Virus: Implications for Vaccination of Island Scrub-Jays (*Aphelocoma insularis*)

Sarah S. Wheeler,¹ Stanley Langevin,¹ Leslie Woods,² Brian D. Carroll,¹ Winston Vickers,³ Scott A. Morrison,⁴ Gwong-Jen J. Chang,⁵ William K. Reisen,¹ and Walter M. Boyce³

Abstract

The devastating effect of West Nile virus (WNV) on the avifauna of North America has led zoo managers and conservationists to attempt to protect vulnerable species through vaccination. The Island Scrub-Jay (*Aphelocoma insularis*) is one such species, being a corvid with a highly restricted insular range. Herein, we used congeneric Western Scrub-Jays (*Aphelocoma californica*) to test the efficacy of three WNV vaccines in protecting jays from an experimental challenge with WNV: (1) the Fort Dodge West Nile-Innovator[®] DNA equine vaccine, (2) an experimental DNA plasmid vaccine, pCBWN, and (3) the Merial Recombitek[®] equine vaccine. Vaccine efficacy after challenge was compared with naïve and nonvaccinated positive controls and a group of naturally immune jays. Overall, vaccination lowered peak viremia compared with nonvaccinated positive controls, but some WNV-related pathology persisted and the viremia was sufficient to possibly infect susceptible vector mosquitoes. The Fort Dodge West Nile-Innovator DNA equine vaccine and the pCBWN vaccine provided humoral immune priming and limited side effects. Five of the six birds vaccinated with the Merial Recombitek vaccine, including a vaccinated, non-WNV challenged control, developed extensive necrotic lesions in the pectoral muscle at the vaccine inoculation sites, which were attributed to the Merial vaccine. In light of the well-documented devastating effects of high morbidity and mortality associated with WNV infection in corvids, vaccination of Island Scrub-Jays with either the Fort Dodge West Nile-Innovator DNA vaccine or the pCBWN vaccine may increase the numbers of birds that would survive an epizootic should WNV become established on Santa Cruz Island.

Key Words: *Aphelocoma*—Conservation—Corvid—Island—Vaccination—West Nile virus.

Introduction

WEST NILE VIRUS (WNV, family *Flaviviridae*, genus *Flavivirus*) has had a devastating effect on North American birds, with hundreds of thousands of fatalities among >200 species (Komar 2003, Kramer et al. 2008). Most impacted have been passerine species within the family *Corvidae*, which have shown significant regional population declines after the invasion of WNV (LaDeau et al. 2007, Wheeler et al. 2009). Especially at risk are species with limited distribution con-

fined to areas with high levels of enzootic WNV transmission; for example, the California endemic Yellow-billed Magpie (*Pica nuttalli*) experienced marked declines upon the arrival of WNV into central California (Crosbie et al. 2008, Wheeler et al. 2009). Mortality associated with WNV has led managers of zoo collections and conservationists to vaccinate vulnerable species (Nusbaum et al. 2003, Chang et al. 2007, Okeson et al. 2007), and in the interest of public health, researchers have investigated the potential for wildlife vaccination to interrupt WNV transmission (Turell et al. 2003, Kilpatrick et al. 2010).

¹Center for Vectorborne Diseases, School of Veterinary Medicine, University of California, Davis, California.

²California Animal Health and Food Safety Laboratory, University of California, Davis, California.

³Wildlife Health Center, School of Veterinary Medicine, University of California, Davis, California.

⁴The Nature Conservancy, San Francisco, California.

⁵Division of Vector-borne Diseases, Department of Health and Human Services, Centers for Disease Control and Prevention, Public Health Services, Fort Collins, Colorado.

The Island Scrub-Jay (*Aphelocoma insularis*), the only insular bird species in North America, occurs only on 255-km² Santa Cruz Island, ~30 km offshore of Santa Barbara, California. This species diverged genetically from the mainland Western Scrub-Jay (*Aphelocoma californica*) >100,000 years ago (Delaney et al. 2008). Due to this restricted range conservationists have had concern that novel pathogens could have catastrophic consequences for this island endemic corvid (Boyce et al. 2011).

WNV was first detected in southern California in 2003 (Reisen et al. 2004) and by the end of 2004 was recorded in every county in the state (Hom et al. 2005). Currently, there is no evidence that WNV has reached Santa Cruz Island (Boyce et al. 2011), indicating that Island Scrub-Jays are naïve to the virus and potentially at high risk in the event of a WNV epizootic. WNV has repeatedly been active in Los Angeles, with outbreaks of human cases in 2004 and 2008, which were associated with massive die-offs of corvids and other passerines (Kwan et al. 2010). WNV activity in the southern coastal region of California increases the risk of WNV introduction to Santa Cruz Island both due to importation of infected mosquitoes and potential introduction by migratory birds.

In response to the threat of WNV introduction onto Santa Cruz Island, conservationists have begun vaccinating a subset of the Island Scrub-Jay population to lessen the extinction risk (Boyce et al. 2011). To address whether vaccination affords protection to Island Scrub-Jays, we evaluated the efficacy of three vaccines to protect the congeneric Western Scrub-Jay from challenge with an isolate of WNV collected from a Yellow-billed Magpie that died in 2004 in Sacramento, California (CA-04) (Deardorff et al. 2006). We elected to conduct this trial with Western rather than Island Scrub-Jays, due to limited population size of the latter, and the assumption that responses between these closely related species would be similar. An experimental host competence study infecting Western Scrub-Jays with the NY99 isolate of WNV resulted in a mean peak viral load >9 log₁₀ plaque forming units (PFU) per mL of sera on 3 and 4 days postinfection (dpi) and 100% mortality (Reisen et al. 2005), indicating that this genus is a highly competent amplifying host and extremely susceptible to WNV-related mortality. Because of the difficulty of capturing and vaccinating free-ranging Island Scrub-Jays more than once per year (Boyce et al. 2011), we chose to evaluate the efficacy of a single inoculation with one of three vaccines to protect Western Scrub-Jays from experimental infection with WNV. Although some of the evaluated vaccines have been tested in other corvids, including Fish Crows (*Corvus ossifragus*) (Turell et al. 2003) and American Crows (*Corvus brachyrhynchos*) (Bunning et al. 2007), this is the first evaluation of these vaccines in the genus *Aphelocoma*.

Materials and Methods

Birds

Western Scrub-Jays were captured in Kern and Yolo counties, CA, banded, and then screened for prior WNV infection by an enzyme immunoassay (Chiles and Reisen 1998, Ebel et al. 2002). Birds were aged by both skull ossification and feather molt patterns (Table 1) (Pyle 1997); all birds included in this experiment had fledged. Birds were housed in screened aviaries at the University of California Arbovirus

Field Station in Bakersfield, CA, until they were moved into individual indoor caging for vaccination. Caging was equipped with perches and provided *ad libitum* with fresh water and Roudybush™ (Davis, CA) High Energy Breeder Diet supplemented with sunflower seeds, cat food, and meal worms.

Vaccines

Three WNV vaccines were evaluated: (1) the Fort Dodge West Nile-Innovator® DNA equine vaccine (Overland Park, KS), which has been used to vaccinate free-ranging Island Scrub-Jays (Boyce et al. 2011), (2) an experimental DNA plasmid vaccine, pCBWN (Davis et al. 2001), which was shown to protect the California Condors (*Gymnogyps californianus*) from WNV infection (Chang et al. 2007), and (3) the Merial Recombitek® WNV equine vaccine (Duluth, GA).

The experimental DNA vaccine pCBWN was developed by the Centers for Disease Control and Prevention, licensed to Fort Dodge, and commercially produced as the Fort Dodge West Nile-Innovator DNA vaccine; both vaccines consisted of a DNA plasmid that expressed WNV premembrane and E (envelope) proteins (Davis et al. 2001, Chang et al. 2004). The pCBWN vaccine was formulated in phosphate-buffered saline at 250 µg and was administered in a single 0.5 mL intramuscular (IM) injection into the pectoral muscle. The Fort Dodge vaccine was formulated with a metastim adjuvant, but the concentration was proprietary. Consultation with the manufacturer's technical staff indicated that the dose required for antigenic effect similar to that of the pCBWN vaccine would require the full 2.0 mL dose recommended for equines. The Fort Dodge vaccine therefore was administered in four 0.5 mL IM injections bilaterally into the pectoral muscle. The Merial vaccine utilized a recombinant canarypox virus (vCP2017) that expressed premembrane and E proteins of the WNV genome (Siger et al. 2006). The Merial vaccine concentration also was proprietary, but the total recommended vaccine dose for equines was 1.0 mL, and this was administered in two 0.5 mL IM injections bilaterally into the pectoral muscle.

Experimental design

Western Scrub-Jays were divided into five groups of six birds each. Three groups were vaccinated by IM inoculation into the pectoral muscle with one of three vaccines as described above (Table 1, groups 1–3); the vaccines were delivered by 28 g syringe. Birds were held in individual cages and sampled for antibody at 1 week prevaccination, and 2 and 4 weeks postvaccination (0.1 mL of blood taken by jugular venipuncture with a 28 g syringe and expelled into 0.9 mL of saline). Experimental group numbers were compromised by active WNV transmission in the Bakersfield area. One bird in group 1 (Fort Dodge) and two birds in group 2 (pCBWN) were WNV antibody positive at prebleed; additionally, a third bird in group 2 died before experimental infection, thereby reducing sample sizes. Group 4 was a positive control group (nonvaccinated) consisting of five birds that tested WNV antibody negative at collection, and were experimentally infected with WNV. Group 5 consisted of five birds that were collected from the wild and WNV antibody positive; they were not vaccinated, and were experimentally challenged with WNV. Group 6 consisted of two birds that were held as

TABLE 1. NEUTRALIZING ANTIBODY TITERS PRE- AND POSTCHALLENGE AND PEAK VIREMIA FOR WESTERN SCRUB-JAYS VACCINATED WITH THREE VACCINES AND THEN CHALLENGED WITH THE CA04 STRAIN OF WEST NILE VIRUS

Group	Vaccine	Bird ^b	Age ^c	Post-vaccine PRNT ₉₀ ^a			Post WNV-Challenge PRNT ₉₀ by dpi							
				2 week	4 week	Peak Viremia ^d	1	2	3	4	5	6	7	14
1	Fort Dodge West Nile-Innovator DNA	6302 (14)	HY	<40 ^e	<40	<2.0	NT	NT	<20 ^f	20	40	320	320	1280
		6303 (7)	U	<40	<40	8.6 (d3)	NT	NT	NT	<20	40	320	NS ^g	NS
		6304 (14)	U	<40	<40	7.2 (d3)	NT	NT	NT	<20	80	320	1280	160
		6305 (14)	AHY	<40	<40	5.2 (d3)	NT	NT	<20	20	160	1280	2560	640
		6306 (14)	HY	<40	<40	7.3 (d4)	NT	NT	NT	<20	<20	80	320	1280
2	pCBWN	6312 (14)	HY	<40	<40	8.4 (d3)	NT	NT	NT	<20	<20	160	320	320
		6313 (14)	HY	<40	<40	4.4 (d4)	NT	NT	NT	<20	40	640	640	1280
		6314 (14)	AHY	<40	<40	5.3 (d3)	NT	NT	NT	<20	320	1280	1280	1280
		6315 (7)	U	<40	<40	8.5 (d3)	NT	NT	NT	<20	80	320	640	NS
		6317 (14)	AHY	<40	<40	7.3 (d3)	NT	NT	NT	<20	20	160	320	640
3	Merial Recombitek	6318 (14)	HY	<40	<40	6.5 (d3)	NT	NT	NT	<20	40	160	320	1280
		6319 (14)	HY	<40	<40	7.3 (d3)	NT	NT	NT	<20	80	160	320	320
		6328 (14)	HY	<40	<40	7.6 (d3)	NT	NT	NT	<20	<20	80	320	320
		6320 ^h (14)	HY	<40	<40	NT	NT	NT	NT	NT	NT	NT	NT	<20
		5528 (14)	AHY	NT	<40	8.8 (d3)	NT	NT	NT	<20	<20	160	80	640
4	Non-Vaccinated WNV-Challenged	5530 (9)	HY	NT	<40	8.3 (d3)	NT	NT	NT	<20	<20	160	160	NS
		5531 (14)	HY	NT	<40	7.4 (d3)	NT	NT	NT	<20	<20	80	320	640
		5532 (6)	HY	NT	<40	8.2 (d3)	NT	NT	NT	<20	40	NS	NS	NS
		5534 (7)	AHY	NT	<40	9.6 (d5)	NT	NT	NT	<20	<20	<20	NS	NS
		6323 (14)	U	>640	>640	<2.0	1280	1280	640	1280	1280	2560	2560	>2560
5	Naturally Immune WNV-Challenged	6325 (14)	AHY	80	160	<2.0	640	1280	1280	2560	1280	1280	1280	>2560
		6326 (14)	HY	80	80	<2.0	320	160	320	320	320	320	640	>2560
		6329 (14)	AHY	320	320	<2.0	1280	640	1280	1280	1280	1280	1280	>2560
		6330 (14)	HY	320	640	<2.0	640	640	640	640	640	640	1280	>2560

^aAll PRNT₉₀ results presented as a reciprocal.

^bUnique bird numbers followed in parentheses by the number of days post WNV infection the bird remained in the study.

^cBirds were aged by skull ossification and feather molt, HY, hatch year; AHY, after hatch year; U, unknown.

^dPeak viremia in log₁₀ PFU/mL followed in parenthesis by the day of detection; 2.0 log₁₀ PFU/mL is the detection limit for this assay.

^eDue to sera dilution at collection, detection limit of assay was 40.

^fSera dilution at collection allowed detection limit of 20.

^gNo sample available.

^h6320 was vaccinated but not WNV-challenged.

dpi, days post infection; WNV, West Nile virus.

negative controls; that is, they were sham-inoculated with saline at vaccination and were not WNV-challenged; this group served as both a handling and a negative control for histopathology and immunohistochemistry (IHC).

Five weeks postvaccination, birds were transported to the California Animal Health and Food Safety laboratory at University of California, Davis where they were housed in Horsfall-Bauer cages, each fitted with its own HEPA filtered negative air system. Birds were housed two to three individuals of the same treatment group per cage, and all birds aside from group 6 (negative controls) and one bird in group 3 (Meriel: bird No. 6320) were challenged by subcutaneous inoculation in the pectoral area with $\sim 2.7 \log_{10}$ PFU per 50 μ L of the CA-04 isolate of WNV. Birds were examined daily for general health and a 0.1 mL blood sample taken by jugular puncture on days 1–7 and expelled into 0.45 mL of virus diluent (Dulbecco's modified Eagle's medium [Gibco Invitrogen, Carlsbad, CA], containing 5% penicillin and streptomycin, and 20% fetal bovine serum), clarified by centrifugation, and then frozen at -80°C until tested for virus and antibody. Birds were held until 2 weeks postinfection at which time they were bled (2.0 mL by jugular puncture), euthanized by CO_2 asphyxiation, and necropsied.

Necropsy and histopathology

Birds were necropsied and samples of brain, spleen, kidney, heart, and pectoral muscle were collected and frozen at -80°C . Samples of brain, heart, liver, kidneys, lungs, spleen, thyroid gland, proventriculus, gizzard, bursa (when present), thymus (when present), pancreas, small intestines, large intestines, adrenal glands, reproductive organs, skeletal muscle, and bone marrow were collected from all birds, immersed in 10% buffered neutral formalin for 24 h, and then embedded in paraffin. Four-micrometer sections of paraffin-embedded tissues were stained with hematoxylin and eosin and examined by light microscopy. Severity of inflammation and necrosis were graded on a scale of 0–5 (0 = absent; 1 = minimal; 2 = mild; 3 = moderate; 4 = severe/multifocal; 5 = severe/diffuse).

Immunohistochemistry

IHC using a WNV polyclonal antibody (BioReliance, Rockville, MD) was used to detect WNV antigen in tissues (Steele et al. 2000, Smedley et al. 2007). Briefly, 4 μ m tissue sections were mounted on positively charged glass slides (Probe-on Plus; FisherBiotech, Pittsburgh, PA), incubated with proteinase K (Dako, Glostrup, Denmark) at room temperature for 6 min, and then followed by serum or peroxidase blocking in a 0.03% H_2O_2 solution at room temperature for 5 min. Primary polyclonal antibody was diluted 1:500 and the subsequent peroxidase-conjugated secondary antibody (En-Vision System; Dako) incubated with the tissue sections at room temperature for 30 min, with phosphate buffered saline washings between incubations. Colorimetric development with 3,*9-3'-diaminobenzidine solution containing H_2O_2 was performed at room temp for 8 min. All immunostained samples were counterstained with hematoxylin and then read by light microscopy. The quantity of infected cells in tissues was graded from 0 to 4 in ten 400 $\times$ fields combined (0 = no staining; 1 = 1–3 labeled cells; 2 = 4–10 diffuse or 1–3 small clusters of labeled cells; 3 = 4 or greater foci with clusters of labeled

cells; 4 = too many to count) (Table 2). Labeled cell types were identified when possible.

Diagnostics

All birds were tested for infectious WNV on 1–7 dpi using a Vero cell plaque assay (Kramer et al. 2002). Sera were serially diluted 10-fold, and 100 μ L pipetted onto six-well plates with confluent Vero cells. A double overlay system was used, with plaques counted 72 h postinfection. Sera were assayed quantitatively for WNV antibody using a plaque reduction neutralization test (PRNT) (Beaty et al. 1995). In brief, sera were heat inactivated at 56°C for 45 min, then serially diluted in a twofold series starting at 1:10. Diluted sera were mixed 1:1 with a virus diluent containing ~ 100 PFU of WNV (CA-04), a double overlay system was used, and end point titers determined as the highest dilution at which 90% of >75 PFU were neutralized (PRNT₉₀).

At necropsy, heart, kidney, brain, spleen, and pectoral muscle tissues from a subset of two birds from groups 1 (Fort Dodge), 2 (pCBWN), and 4 (unvaccinated WNV-challenged) were screened for WNV RNA by quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR) and virus isolation attempted (Table 2). Individuals for this subset were chosen based on predominately negative IHC results at 14 dpi. These extra tests were performed to determine if IHC results were negative due to viral clearance or potential limitations in our IHC assay. Tissues were homogenized in virus diluent using a mixer mill (MM300; Retsch, Haan, Germany), with one aliquot used for virus isolation and a second for qRT-PCR. Virus isolation was attempted by Vero cell plaque assay after blind passage on C6/36 *Aedes albopictus* cells for 7 days. RNA was extracted using the RNeasy lipid tissue mini kit (Qiagen, Valencia, CA), and qRT-PCR performed using previously published methods and primers specific for the envelop region of the viral genome (Lanciotti et al. 2000) using a 7900 TaqMan platform (Applied Biosystems, Carlsbad, CA).

Statistical analyses

Percent dying per group were compared by contingency Chi square (Hintze 1998). Viremia estimates as $\log_{10}$ PFU/mL were compared among groups 1–4 and days 1–7 postinoculation by repeated measures analysis of variance (ANOVA) (Hintze 1998). One-way ANOVAs using peak viremias on 3 dpi were used to test for differences between vaccinated and control birds and for differences among vaccinated birds. PRNT₉₀ end-point titers were inverted, transformed by $\ln(y+1)$, and vaccinated groups 1–3 compared with unvaccinated control group 4 using a *t*-test for unequal variances (Hintze 1998). Means were presented as back transformed or geometric mean titers.

Ethics

The collection, housing, vaccination, transport, and infection of Western Scrub-Jays were conducted under approved University of California, Davis, IACUC protocols 13012, 12876, and 12880. Birds were collected by grain-baited trap and mist net (USGS Master Station Banding Permit 22763) under State of California Scientific Collecting Permits and taken for experimentation under Federal Permit MB082812. BSL3 laboratory facilities were approved under BUA 0873

TABLE 2. IMMUNOHISTOCHEMISTRY, LESION SEVERITY, QUANTITATIVE REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION, AND VIRUS ISOLATION FINDINGS FOR VACCINATED AND NONVACCINATED WESTERN SCRUB-JAYS CHALLENGED WITH THE CA04 ISOLATE OF WEST NILE VIRUS

Group	Vaccine	Bird	IHC ^a			Lesion severity ^b					qRT-PCR ^c					Virus isolation ^d						
			Heart	Kidney	Spleen	Brain	Pectoral	Heart	Kidney	Spleen	Brain	Pectoral	Heart	Kidney	Spleen	Brain	Pectoral	Heart	Kidney	Spleen	Brain	Pectoral
1	Fort Dodge West Nile-Innovator DNA	6302 (d14)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		6303 (d7)	2	3	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		6304 (d14)	0	1	1	0	0	3	2	3	0	4	0	4	0	4	0	0	0	0	0	0
		6305 (d14)	0	0	0	0	0	0	2	0	0	3	0	3	20	26	— ^e	24	—	—	—	—
		6306 (d14)	0	0	0	1	0	0	2	0	0	2	0	0	0	0	0	0	0	0	0	0
2	pCBWN	6312 (d14)	0	0	0	0	0	0	4	3	3	3	3	0	0	0	0	0	0	0	0	0
		6313 (d14)	0	0	0	0	0	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0
		6314 (d14)	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0
		6315 (d7)	3	2	4+	0	4++++	2	0	4	0	4	0	4	0	0	0	0	0	0	0	0
3	Merial Recombitek	6317 (d14)	1	1	0	0	0	2	4	2	0	0	0	0	0	0	0	0	0	0	0	0
		6318 (d14)	0	0	0	0	0	0	2	0	0	0	2	2	0	0	0	0	0	0	0	0
		6319 (d14)	0	2	1	0	0	4	3	2	3	2	3	2	5	0	0	0	0	0	0	0
		6328 (d14)	0	1	0	0	0	2	2	2	2	2	1	4	0	0	0	0	0	0	0	0
		6320 (neg)	0	0	0	0	0	0	0	0	0	2	0	0	5	0	0	0	0	0	0	0
		5528 (d14)	0	0	0	0	0	0	4	2	2	0	0	2	—	19	20	29	25	—	+	—
4	Nonvaccinated WNV-challenged	5530 (d9)	4	3	2	3	3	3	0	0	0	0	3	0	0	0	0	0	0	0	0	0
		5531 (d14)	0	2	1	1	0	2	2	0	2	0	2	3	26	26	21	29	31	—	—	—
		5532 (d6)	3	3	3	0	4	1	0	0	0	0	0	3	0	0	0	0	0	0	0	0
		5534 (d7)	4	3	4+	2	4	1	0	3	0	3	0	3	0	0	0	0	0	0	0	0
		6331	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0
6	Negative controls	6332	0	0	0	0	0	0	2	2	2	0	0	0	0	0	0	0	0	0	0	

^aImmunohistochemistry grading: 0, no cells labeled; 1, 1–3 cells labeled; 2, 4–10 cells diffuse or 1–3 small clusters of cells labeled; 3, multifocal (4 or greater foci with multiple cells labeled); 4, too many to count.

^bHistopathology lesion severity scores: 1, minimal; 2, mild; 3, moderate; 4, severe.

^cqRT-PCR TaqMan ct scores rounded to the nearest whole number.

^dVirus isolation results: “—,” no infectious virus isolated; “+,” infectious virus isolated.

^e“—,” no viral RNA detected.

qRT-PCR, quantitative reverse transcriptase–polymerase chain reaction.

approved by the University of California, Davis, Environmental Health and Safety Committee and USDA Permit 47901.

Results

Mortality

Birds were marginally protected from mortality by vaccination when compared with unvaccinated experimentally infected control group 4 ($\chi^2 = 3.2$, $df = 1$, $p = 0.079$, Table 1). Three of five unvaccinated controls died on days 6–9, whereas one bird vaccinated with Fort Dodge (No. 6303, Table 1) and one bird vaccinated with Merial (No. 6315, Table 1) died 7 dpi. All birds that were antibody positive at capture (group 5) survived challenge, as did both of the negative controls (group 6), indicating that our husbandry and sampling were not likely the cause of observed mortality.

Viremia

One bird (No. 6301) vaccinated with Fort Dodge (group 1) failed to develop a viremia. This bird had a much lower antibody titer (1:20) on 4 dpi compared with the naturally infected birds in group 5 (range: 1:320 to >1:640; Table 1), indicating that it probably was not naturally infected before challenge. In addition, this bird did not have a WNV-specific neutralizing antibody response before challenge. While this bird was a singular case of sterilizing immunity postvaccination, it was considered to be an experimental outlier and was not included in our viremia profile analysis. When compared by repeated measures ANOVA, there were no significant differences ($p > 0.05$) among vaccinated and control groups; however, time ($F = 45.9$, $df = 6, 18$, $p < 0.001$), and the time $\times$ vaccine treatment groups ($F = 2.0$, $df = 18, 74$, $p = 0.02$) were significantly different. Because time $\times$ vaccine interaction term was significant, groups were compared using data from day 3 when viremia peaked for most birds (Fig. 1). The unvaccinated control group 4 had a significantly ($F = 7.1$, $df = 1, 15$, $p = 0.04$) greater mean viremia (mean = $8.3 \log_{10}$ PFU/mL) than vaccinated birds in groups 1–3 (pooled mean = $6.8 \log_{10}$ PFU/mL); there were no significant differences ($p > 0.05$) among mean viremias on day 3 for the vaccinated birds in groups 1–3. All five of the naturally infected

birds (group 5) exhibited sterilizing immunity and no viremias were detected on days 1–3 pi (Fig. 1).

Antibody response

After vaccination, birds were sampled for antibody at 2 and 4 weeks. Naturally infected birds that were antibody positive at capture had PRNT₉₀ titers, which ranged from 1:80 to >1:640 at both 2 and 4 weeks. As found with the Island Scrub-Jays vaccinated with a single dose of either the Fort Dodge West Nile-Innovator killed or DNA vaccines (Boyce et al. 2011) and American Robins vaccinated with pCBWN (Kilpatrick et al. 2010), none of the vaccinated Western Scrub-Jays developed an antibody response before infection detectable by enzyme immunoassay or PRNT₉₀ at a dilution of 1:20.

Birds were tested for antibody titer by PRNT₉₀ on 4–7 and 14 dpi (Fig. 2, Table 1). Neutralizing antibody was first detected on 4 dpi in two birds vaccinated with Fort Dodge (group 1). As noted above, one of these birds (group 1, No. 6302) was antibody negative at prebleed and at weeks 2 and 4 postvaccination, failed to produce a WNV viremia postchallenge, but showed a strong protective antibody response at 14 dpi. This bird appears to be a singular case of sterilizing immunity due to vaccination with Fort Dodge. On 5 dpi, vaccinated birds in groups 1 (Fort Dodge) and 3 (Merial) showed a significant ($t > 3.1$, $p = 0.03$) increase in PRNT titer compared with group 4 (nonvaccinated, WNV-challenged); group 2 (pCBWN) was not significantly different ($p = 0.14$) (Fig. 2). On days 6 and 7, vaccinated groups 1 (Fort Dodge) and 2 (pCBWN), but not group 3 (Merial), had titers significantly greater ($t > 2.12$, $p < 0.03$) than group 4 (nonvaccinated, WNV-challenged) (Fig. 2). By 14 dpi there were no significant differences ($p > 0.3$) among titers for all groups. Collectively, these data indicated that the vaccinated birds (especially those vaccinated with the Fort Dodge West Nile-Innovator, group 1) were immunologically primed and able to more rapidly produce neutralizing antibody than were the nonvaccinated positive control birds.

Gross necropsy findings

At necropsy, two of the five birds vaccinated with Fort Dodge (group 1) had pale foci on the ventral surface of the pectoral muscle consistent with subcutaneous WNV inocu-

FIG. 1. Mean viremia in $\log_{10}$ plaque forming units (PFU) per mL for vaccinated (dashed lines) and unvaccinated (solid lines) Western Scrub-Jays challenged with WNV CA04. Minimal threshold of detection was $2 \log_{10}$ PFU/mL. WNV, West Nile virus.

FIG. 2. Endpoint WNV PRNT₉₀ titers for vaccinated (dashed lines) and unvaccinated (solid line) Western Scrub-Jays challenged with the CA04 strain of WNV.

FIG. 3. Pectoral muscle of Western Scrub-Jay (bird No. 6328, group 3). Whole pectoral muscle showing bilateral lesions attributed to vaccination with Merial Recombitek WNV vaccine.

lation overlying this site, and the one bird (No. 6303) that died 7 dpi had multifocal pale lesions in the heart. One of three birds (No. 6312) vaccinated with pCBWN (group 2) also had a pale focus in the pectoral muscle below the WNV inoculation site, but no other significant gross lesions were noted. Five of the six birds vaccinated with Merial (group 3), including the vaccinated, non-WNV challenged control (No. 6320), developed extensive necrotic lesions bilaterally in the pectoral muscle at the vaccine inoculation sites (Figs. 3 and 4). No other gross lesions were seen in group 3 and similar necrotic lesions in the pectoral muscle were not seen in any other group. Of the birds in group 4 (nonvaccinated, WNV-challenged), 1 of 5 (No. 5530) had a pale focus in the pectoral muscle below the WNV inoculation site, and multiple pale foci in the heart (Fig. 5).

Histopathology findings

Significant lesions attributable to WNV were seen in WNV-challenged birds and are described by group (1–5). The negative controls (group 6) did not have any lesions in the brain,

FIG. 4. Pectoral muscle of Western Scrub-Jay (bird No. 6328, group 3). Cut pectoral muscle exhibiting the depth of necrosis attributed to vaccination with Merial Recombitek WNV vaccine.

FIG. 5. Heart of Western Scrub-Jay (bird No. 5530, group 4). Multifocal pale foci in the heart are congruent with myocardial necrosis and inflammation.

heart, bone marrow, or spleen; one bird had a single vessel with vasculitis in the mesentery. Table 2 contains a synopsis of lesion severity in the primary organs affected by WNV.

Birds vaccinated with Fort Dodge (group 1, $n = 5$) had lesions in the heart, nervous system, liver, spleen, bone marrow, and pectoral muscle. Specifically, three birds had myocardial degeneration and inflammation accompanied by variable mononuclear cell infiltrates. One bird had gliosis in the molecular layer of the cerebellum and another had lymphocytic neuritis of Auerbach's plexus. Hepatitis was evident in one bird that was very similar to natural cases of WNV characterized by Kupffer cell hypertrophy and necrosis, sinusoidal leukocytosis, and necrosis of periportal leukocytes. Two birds had lymphocellular necrosis in the spleen and/or bone marrow. Four birds had lesions in the pectoral muscle, characterized by acute, mild-to-moderate hyalinization of the sarcoplasm and contraction band necrosis (Fig. 6), these lesions were mostly inflammatory in nature.

FIG. 6. Pectoral muscle of Western Scrub-Jay (bird No. 6304, group 1). Myositis associated with WNV inoculum. Scattered hyalinization of myocytes. Note preservation of myocytes (H&E). H&E, hematoxylin and eosin.

FIG. 7. Brain of Western Scrub-Jay (bird No. 6318, group 3). Encephalitis characterized by gliosis in the molecular layer of the cerebellum (H&E).

Birds vaccinated with pCBWN (group 2, $n = 3$) had lesions in the nervous system, pectoral muscle, and vasculature. Specifically, in one bird gliosis was evident in the molecular layer of the cerebellum, and one bird had myositis of the pectoral muscle without necrosis. Systemic vasculitis was seen in a single bird affecting spleen, liver, carotid artery, thyroid, adductor muscle, heart, kidneys, and gastrointestinal serosal vessels.

Birds vaccinated with Merial (group 3, $n = 6$) had lesions in the heart, nervous system, spleen, bone marrow, vasculature, and pectoral muscle. Specifically, five birds had myocarditis/ myocardial necrosis: three with mild lesions and two with moderate lesions. Four birds had gliosis and perivascular lymphocytic inflammation in the brain (encephalitis) and peripheral nerves. In all of these cases, lesions in the brain were limited to the molecular layer of the cerebellum (Fig. 7) with rare foci in the brain stem. Three birds had lymphocellular necrosis in the spleen or bone marrow and three had

FIG. 9. Pectoral muscle of Western Scrub-Jay (bird No. 6315; group 3). Low magnification demonstrates extensive caseous necrosis in the pectoral muscle associated with the Merial Recombitek WNV vaccine (H&E).

vasculitis (Fig. 8) in several tissues, including skeletal muscle, spleen, kidneys, mesentery, serosa of the proventriculus, gizzard or intestine, and heart. Severe coagulation necrosis in the pectoral muscle (Figs. 9 and 10) was seen in all birds vaccinated with Merial, inflammation was less prominent, with variable combinations and quantities of pleocellular infiltrates that included multinucleated giant cells, macrophages, lymphocytes, plasma cells, and heterophils. These pectoral lesions were strikingly different from the pectoral lesions seen in any other group particularly in the type and severity of necrosis.

The nonvaccinated WNV-challenged birds (group 4, $n = 5$) had lesions in the heart, nervous system, spleen, bone marrow, vasculature, and pectoral muscle. Specifically, all five birds had degenerative and inflammatory myocarditis (Fig. 11): three cases were mild and two were moderate. Nervous system lesions included: gliosis in the molecular

FIG. 8. Heart of Western Scrub-Jay (bird No. 6317, group 3). Vasculitis with fibrinoid necrosis and interstitial pleocellular inflammation in adjacent myocardium (H&E).

FIG. 10. Pectoral muscle of Western Scrub-Jay (bird No. 6315; group 3). Higher magnification demonstrates caseous necrosis with dystrophic calcification. Note there is little preservation of muscle cells (H&E).

FIG. 11. Heart of Western Scrub-Jay (bird No. 5530, group 4). Lymphoplasmacytic myocarditis with multifocal degeneration (H&E).

layer of the cerebellum with associated focal meningitis in one bird, ganglioneuritis in the gastrointestinal tract in two birds, and neuritis in two birds. Lymphocellular necrosis and/or depletion, some with fibrin lakes, was seen in the bone marrow, spleen, or bursa in four birds. The organs affected in one bird with systemic vasculitis included spleen, liver, heart, peripheral nerves, proventriculus, and gizzard. All five had lesions in the pectoral muscle characterized by interstitial perivascular lymphocytes, similar to the lesions seen in the group 1 birds.

Immunohistochemistry

Immunolabeled WNV-infected cells were detected in the heart, kidney, spleen, and pectoral muscle in all birds that died acutely at 6–9 dpi, including one of five birds in both group 1 (Fort Dodge) and group 3 (Merial), and three of five

FIG. 12. Spleen of Western Scrub-Jay (bird No. 6315, group 3). Immunohistochemistry of the spleen demonstrates heavy diffuse infection of macrophages (presumptive) and endothelial cells in both blood vessels.

FIG. 13. Brain of Western Scrub-Jay (bird No. 5530, group 4). Immunohistochemistry (WNV polyclonal antibody) of the brain demonstrates WNV-infected Purkinje cells and dendritic processes.

birds in group 4 (nonvaccinated WNV-challenged) (Table 2). Infected cells included cardiocytes, interstitial cells, endothelial cells and leukocyte infiltrates in the heart, renal tubular epithelium and leukocytic infiltrates in the kidneys, macrophages (Fig. 12) and endothelial cells in the spleen, myocytes interstitial cells and leukocytes in the skeletal muscle and Purkinje cells, and dendritic processes in the brain (Fig. 13). Target cells and tissues did not differ between vaccinated and nonvaccinated birds. Immunolabeling in birds sacrificed 14 dpi was lower-grade than immunolabeling in birds that died acutely, but was detected in three of four birds in group 1 (Fort Dodge) and group 3 (Merial), and one of two birds in group 4 (nonvaccinated WNV-challenged). Table 2 shows the intensity of IHC staining as compared with lesion severity, virus isolation, and qRT-PCR findings. The lack of staining in birds sacrificed on 14 dpi could be explained by a reduction in WNV protein due to clearance of infection and/or rising WNV-specific antibody levels competing with the IHC primary antibody for immunogenic epitopes on the viral envelope protein. Other labeled cells in tissues not listed in Table 2 included peripheral nerves, bone marrow, lungs, sinusoidal leukocytes and Kupffer cells in the liver, thecal cells of follicles in the ovary, leukocytes in the lamina propria and crypt epithelial cells in the intestines, leukocytes and glandular epithelium in the proventriculus and gizzard, islet cells and interstitial leukocytes in the pancreas, and adipocytes in the mesentery.

Discussion

Wild birds maintained in zoos and outdoor aviaries, as well as free-ranging species like California Condors (Chang et al. 2007) and now Island Scrub-Jays (Boyce et al. 2011), are routinely vaccinated for protection against WNV virus. However, there are no WNV vaccines approved specifically for use in birds, and there are few data on the efficacy of commercially available equine vaccines in birds. In our study, none of the three vaccines provided the same level of protection upon challenge as a naturally mounted immune response after acute infection with WNV, exemplified by the

naturally infected birds in group 5. However, because WNV infection in highly susceptible species such as corvids is often fatal (Komar et al. 2003, Reisen et al. 2005), as seen in our nonvaccinated positive controls (group 4) of which 60% succumbed to infection, even a partially effective vaccine may be beneficial in vulnerable populations.

The Fort Dodge West Nile-Innovator DNA and pCBWN provided the best immune-priming and had the lowest peak viremias. Because the pCBWN vaccine group had low sample sizes, a direct comparison between groups 1 and 2 lacked statistical power. All of the birds vaccinated with Merial developed bilateral necrotic lesions in the pectoral muscle at the vaccination site. These lesions were apparent at gross necropsy (Figs. 3 and 4) in all but one bird, including the bird (No. 6320) that was vaccinated but not challenged with WNV. On gross and microscopic examination, Merial-vaccinated birds had extensive coagulation necrosis extending deep into the pectoral muscle (Figs. 5 and 6). These lesions were attributed to the Merial vaccine, because they were seen in all Merial-vaccinated birds, including the vaccinated nonchallenged bird (No. 6320), and were not seen in any other group. In mammalian hosts and poultry, recombinant vaccines created from *Avipox* genera, such as fowlpox and canarypox, have been considered safe and immunogenic (Taylor et al. 1988, 1991), and the WNV canarypox vaccine has been shown to be safe and effective in horses, dogs, and cats (Minke et al. 2004, Karaca et al. 2005). The lesions noted at the vaccine inoculation sites in our study may have been due to replication of the recombinant canarypox virus, and we recommend that the Merial vaccine be assessed carefully before its use in other avian species, especially passerines. Although some immune-priming was detected in this group and the overall viremia was somewhat lower than the nonvaccinated positive controls, the vaccine was not as immunogenic as the Fort Dodge vaccine. While pectoral lesions were also noted in the group 1 and 4 (Table 2), these lesions were predominately inflammatory and acute in nature. The inflammatory component was attributed to subcutaneous WNV challenge inoculation over the pectoral muscle, as seen in previous experimental WNV infection studies (L. Woods, personal observation).

Each vaccine was evaluated to discern whether it was protective against WNV infection and if vaccine-related tissue damage would affect survivability in free-ranging birds. Two birds vaccinated with Fort Dodge had lesions that may have affected survivability, one with a systemic vasculitis and moderately severe myocarditis and one with encephalitis. Encephalitis and systemic vasculitis also were detected in one bird vaccinated with pCBWN. Four birds vaccinated with Merial, which were sacrificed 14 dpi, had lesions in target tissues that were typical of WNV infection, including encephalitis, polyneuritis, splenitis, and myocarditis/myocardial degeneration. Although these birds did not die during our study, lesions detected in these birds may have impacted survival in nature. Three birds from the Merial group had a systemic vasculitis with fibrinoid necrosis in vessel walls in the heart, kidney, spleen, and mesentery. IHC did not reveal any deposition of antigen in the vessel walls, which suggests that the vasculitis may have been caused by a type III hypersensitivity immune complex reaction. These factors, coupled with pectoral muscle necrosis induced by the Merial vaccine, would certainly have had significant impact on the survivorship of these vaccinated birds.

We were unable to detect a postvaccination antibody response in any of the vaccinated birds before WNV challenge. These results differed from previous studies that utilized pCBWN with multiple vaccinations and lower PRNT cut-off values. In one study (Bunning et al. 2007), where American Crows received two vaccinations at 21-day intervals, 80% of the birds were PRNT₇₀ positive for WNV antibodies at a serum dilution of 1:10 six weeks postvaccination. However, by 9 weeks postvaccination the percent PRNT₇₀ antibody positive dropped to 50%. In a second study (Turell et al. 2003), where Fish Crows received a single vaccination, 56% of the birds developed a PRNT₈₀ detectable antibody response at a serum dilution of 1:20 by 14 days postvaccination; however, by day 42 postvaccination antibodies were no longer detectable at PRNT₈₀. In agreement with our findings, American Robins vaccinated with the pCBWN vaccine also failed to produce detectable antibodies when given a single vaccination and tested by PRNT₉₀ at a serum dilution of 1:10, 14 days postvaccination (Kilpatrick et al. 2010). Likewise, antibodies were not detected in 10 free-ranging Island Scrub-Jays that were vaccinated a single time with the Fort Dodge West Nile-Innovator DNA vaccine (Boyce et al. 2011). Jays may need multiple inoculations or "boosters" with DNA vaccines to elevate antibody titers to detectable levels, similar to what was observed when free-ranging Island Scrub-Jays were inoculated with a killed vaccine (Fort Dodge West Nile-Innovator). Boyce et al. (2011) found that five jays vaccinated twice with the killed vaccine had detectable PRNT₈₀ antibody titers >1:20, whereas antibodies were detected in only 1 of 13 jays that received a single dose of killed vaccine. The lack of sustained humoral responses in WNV-vaccinated Island Scrub-Jays (single dose), as opposed to the responses of mice and House Sparrows experimentally exposed to wild-type WNV (Nemeth et al. 2009, Appler et al. 2010), illustrates the challenge inherent in designing efficacious vaccines for certain vertebrate species. These results also stress the importance of immunogenic epitopes located on WNV nonstructural proteins and the propagation of competent virus to stimulate a long-lasting sterile immunity in birds.

Aside from the one bird (No. 6302) in group 1 that apparently developed sterilizing immunity and one bird (No. 6313) in group 2 that had a reduced viremia, all vaccinated birds produced viremias suitable to infect California *Culex* vectors with WNV (Reisen et al. 2005, 2008). Therefore, although vaccination with all three vaccines lowered the viremia compared with nonvaccinated positive controls, the viremia was not lowered sufficiently to preclude the vaccinated birds from participating in the WNV transmission cycle. These findings suggest that vaccination of free-ranging Island Scrub-Jays with the vaccines we evaluated would reduce, but not effectively interrupt WNV transmission.

In summary, none of the vaccines we tested elicited sterilizing immunity after one vaccination, and none were completely without side effects. The Fort Dodge and pCBWN vaccines provided the best protective immune priming with the least side effects. In light of the well-documented devastating effects of high morbidity and mortality associated with WNV infection in corvids, vaccination with either vaccine could increase the numbers of birds that survive an epizootic should WNV be introduced to Santa Cruz Island. Unfortunately, neither of these vaccines are readily available for use in wild birds. The Fort Dodge vaccine was removed from the commercial market in

2010 after we completed this study, and pCBWN experimental vaccine is only available in limited quantities. In contrast, the Merial vaccine remains commercially available, but caused unacceptable side effects and appeared to be less effective. We acknowledge the limitations of our study (especially sample sizes), and encourage additional work to evaluate the efficacy and side effects of WNV vaccines for use in wild birds.

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Disclosure Statement

No competing financial interests exist.

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Address correspondence to:

Walter M. Boyce
Wildlife Health Center
School of Veterinary Medicine
University of California
Old Davis Road
Davis, CA 95616

E-mail: wmboyce@ucdavis.edu