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SUSCEPTIBILITY OF GREATER SAGE-GROUSE TO EXPERIMENTAL INFECTION WITH WEST NILE VIRUS

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ABSTRACT: Populations of greater sage-grouse (*Centrocercus urophasianus*) have declined 45–80% in North America since 1950. Although much of this decline has been attributed to habitat loss, recent field studies have indicated that West Nile virus (WNV) has had a significant negative impact on local populations of grouse. We confirm the susceptibility of greater sage-grouse to WNV infection in laboratory experimental studies. Grouse were challenged by subcutaneous injection of WNV ($10^{3.2}$ plaque-forming units [PFUs]). All grouse died within 6 days of infection. The Kaplan-Meier estimate for 50% survival was 4.5 days. Mean peak viremia for nonvaccinated birds was $10^{6.4}$ PFUs/ml ($\pm 10^{0.2}$ PFUs/ml, standard error of the mean [SEM]). Virus was shed cloacally and orally. Four of the five vaccinated grouse died, but survival time was increased (50% survival=9.5 days), with 1 grouse surviving to the end-point of the experiment (14 days) with no signs of illness. Mean peak viremia for the vaccinated birds was $10^{2.3}$ PFUs/ml ($\pm 10^{0.6}$ PFUs/ml, SEM). Two birds cleared the virus from their blood before death or euthanasia. These data emphasize the high susceptibility of greater sage-grouse to infection with WNV.

Key words: *Centrocercus urophasianus*, experimental infection, greater sage-grouse, vaccine, West Nile virus.

INTRODUCTION

Populations of greater sage-grouse (*Centrocercus urophasianus*) have declined 45–80% in North America since 1950 (Braun, 1998). It is believed that the primary cause for population decline is loss of habitat owing to cultivation and overgrazing. As a consequence, Gunnison sage-grouse (*Centrocercus minimus*) and Western sage-grouse (*Centrocercus urophasianus phaios*) are under consideration for listing as threatened species (United States Fish and Wildlife Service, 2004).

Additional pressures on sage-grouse populations have become apparent. During 2003, telemetry studies on sage-grouse revealed unusual mortality of marked birds. Necropsies revealed that these clusters of mortality were attributable to West Nile virus (WNV) infection (Naugle et al., 2004). These observations and the spread of WNV across the continent have raised additional concerns among wildlife managers relative to the well-being of sage-grouse populations. The susceptibil-

ity, mortality, and risks of sage-grouse populations to WNV infection are critical questions to resolve to determine what management options might be available for the species' conservation.

The objectives of this study were to compare viremia and survivorship between vaccinated and nonvaccinated greater sage-grouse subjected to experimental challenge with WNV.

MATERIALS AND METHODS

Study subjects

Twenty-one greater sage-grouse were trapped in the Sheldon National Wildlife Refuge, northwestern Nevada, USA, by the United States Fish and Wildlife Service in October 2003. Birds were banded, and blood was collected from each individual via ulnar venipuncture. The grouse were transported by plane in 7 carriers (66×35×40 cm) on the same day to Fort Collins, Colorado, USA, and driven by truck to the United States Department of Agriculture–National Wildlife Research Center. Upon arrival at the Research Center, all grouse were determined to be hatch-year birds by weight and plumage

characteristics. Grouse were dusted with Drione® (Bayer Environmental Science, Montvale, New Jersey, USA) to control feather mites, and wing feathers were clipped to reduce cage trauma. Grouse were held in captivity until experimentation (Oesterle et al., 2005).

Experimental design

The principal goals of the experiment were to describe the time course of infection and survivorship in greater sage-grouse experimentally challenged with West Nile virus (WNV) as a function of vaccination status. Nine grouse were assigned to the nonvaccinated WNV infection group, and 5 birds were assigned to the vaccinated WNV infection group. One bird was used as a negative control (i.e., sham infection/sham vaccination).

Vaccine

A proprietary experimental DNA vaccine was provided by Merial Ltd., Athens, Georgia, USA. This vaccine was made by inserting the DNA complementary to the WNV structural genes prM and E into a fowl pox DNA backbone. The frozen vaccine was thawed, and 0.2 ml was diluted with 4.8 ml polymer adjuvant (Carbopol, Merial Ltd.). Then 0.2 ml vaccine or Carbopol was delivered subcutaneously at the inguinal fold of the left leg, resulting in vaccination of a 10^7 tissue culture infective dose (TCID₅₀) per bird. An identical booster vaccination was delivered 21 days after the initial vaccination. No apparent necrosis or other overt effects of vaccination or sham treatment were observed.

Experimental infection

Grouse were challenged 35 days postvaccination by subcutaneous injection in the inguinal fold with $10^{3.2}$ plaque-forming units (PFUs) WNV (NY-99-6625, 1 passage in Vero cell culture, isolated from crow brain) delivered in 0.1 ml saline. Virus was diluted in BA-1 and titer verified by plaque assay on Vero cells at the time of inoculation. This dose was deemed to be within the range of documented viral load in mosquito saliva and was the approximate equivalent infectious dose delivered by 1 to 6 mosquito bites ($10^{1.3}$ – $10^{3.8}$ PFU equivalents; Bunning et al., 2002; Vanlandingham et al., 2004). During the postinfection period, blood from grouse was collected daily to quantify viremia (up to the point of death, or the end-point of the experiment, i.e., 14 days). Cloacal and oral swabs (sterile, cotton-tipped) also were col-

lected daily to check for shed virus and placed in 1 ml of BA-1.

Statistical analysis

Probability of survivorship was calculated using the Kaplan-Meier product limit estimate for censored data, and comparison of survivorship estimates between experimental groups (vaccinated versus not vaccinated) was made using Cox's F-test (StatSoft, 1999). All error estimates are reported as ± 1 standard error of the mean (SEM).

Enzyme-linked immunosorbant assays

To detect flavivirus-specific immunoglobulin M (IgM) antibodies, an IgM capture enzyme-linked immunosorbant assay was used (cELISA; Johnson et al., 2003). WNV positive and negative antigens were obtained from the Centers for Disease Control and Prevention, Atlanta, Georgia, USA, and made following the method of Clarke and Casals (1958). Test samples were analyzed in duplicate, with 3 wells of control chicken serum included on each plate. Positive-to-negative ratios (P/N) were calculated as the mean optical density (OD) of the test or positive control serum wells divided by the mean OD of the negative control serum wells. The criterion for a valid test was a P/N for the positive control > 2.0 .

To detect flavivirus-specific immunoglobulin G (IgG) antibodies, an indirect ELISA (iELISA; Ebel et al., 2002) was used. The iELISA positive and negative antigens were provided by the New York State Department of Health's Wadsworth Center Health Laboratory, Albany, New York, USA (Ebel et al., 2001). Test samples were analyzed in duplicate, with 3 wells of control chicken serum included on each plate. The calculation and criterion for a positive test were as described above.

An epitope-blocking assay (bELISA; Blitvich et al., 2003) was used to detect all WNV-specific antibodies (IgG, IgM, etc.) in a sample. We used unlabeled monoclonal antibody (MAb) 3.112G (Chemicon, Temecula, California), which is specifically reactive to WNV, offers a high degree of sensitivity, and enables the assay to be taxon-independent in its ability to detect antibodies against WNV (Blitvich et al., 2003). The MAb 3.112G detects the NS-1 epitope (Hall et al., 1990, 1991). Interpretation of the test was based on the following criterion and calculation. The percentage inhibition of MAb 3.112G binding was calculated as $100 - [(TS - B)/(CS - B)] \times 100$, where TS is the mean optical density of the test serum, CS is the mean optical density of the control

serum (from uninfected chickens), and B is the background optical density. Test samples were analyzed in duplicate, with 3 wells of control chicken serum included on each plate. The percentage inhibition was calculated once the mean OD in the wells containing the control serum samples exceeded 0.3. An inhibition value of >30% was considered to indicate the presence of WNV antibodies (Blitvich et al., 2003).

Reverse transcription polymerase chain reaction assay

WNV RNA was extracted from serum (Lanciotti et al., 2000) and oral and cloacal swabs (Komar et al., 2002) using the QIAamp® Viral RNA Mini Kit (QIAGEN, Valencia, California, USA). The Taqman® One-Step reverse transcriptase polymerase chain reaction (RT-PCR) system (Applied Biosystems, Foster City, California) using primers and probes based on the published sequence of the NY99 strain of WNV (GenBank accession number AF196835) and the method of Lanciotti et al. (2000) were used to quantify WNV RNA in samples.

Standard curves were generated using serial dilutions ($10\times$) of WNV stock (10^7 PFUs/ml, verified by plaque assay). RNA from each dilution (10^6 – 10^{-3}) was extracted as described above. In our laboratory, the detection limit of the Applied Biosystems PCR using the Taq-Man RT-PCR for WNV detection is $10^{-1.1}$ PFU equivalents/ml. This is comparable to published reports by other laboratories (Lanciotti et al., 2000). The RT-PCR method quantifies WNV RNA in the serum sample. Measures of viremia reported herein are in terms of PFU equivalents based on the conversion described above.

RESULTS

After arrival from the field (October 2003), initial serum samples indicated all grouse were negative for flavivirus immunoglobulin G (IgG) antibodies (Fig. 1). During April 2004, another serum sample was obtained before vaccination treatment. Again, all birds were negative for IgG flavivirus antibodies. Birds also tested negative for immunoglobulin M (IgM) and IgG antibodies to West Nile virus (WNV) at both time intervals as determined by the epitope-blocking assay (bELISA).

The fowl pox vaccine used in this study

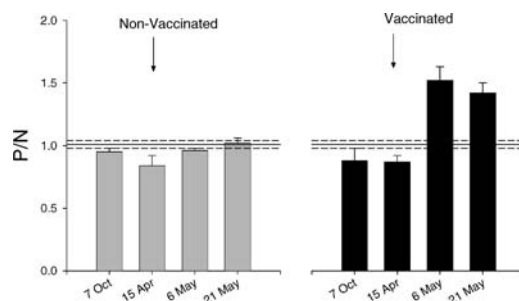


FIGURE 1. Mean (\pm SEM) P/N for flavivirus-specific IgG antibodies as assayed by iELISA as a function of time and vaccination status for greater sage-grouse. Arrows indicate the date at which vaccine or sham injection was administered. Horizontal lines depict mean (\pm SEM) WNV negative chicken serum control P/N values for the iELISA. High-titer, positive control chicken serum P/N values (mean = 2.93 ± 0.20) are not shown.

did not contain the NS-1 gene of WNV. As a consequence, we were unable to detect the antibody response after vaccination using the bELISA (MAb 3112.G is specific to NS-1). In its stead we used the indirect ELISA (iELISA) to detect IgG antibodies against WNV in the fowl pox-vaccinated group of grouse. Twenty-one days postvaccination, the vaccinated group showed elevated levels for flavivirus-specific IgG antibodies (Fig. 1). The positive-to-negative (P/N) values for flavivirus-specific IgG antibodies to flavivirus remained stable at 35 days postinoculation. However, the amount of antibody was substantially lower than the high antibody titer for positive control chicken serum ($P/N = 3.2 \pm 0.2$). WNV antibodies in the vaccinated group also were monitored using the bELISA. All of these assays were negative, consistent with interpretation that the vaccine construct included the envelope-coding region, but not the NS-1-coding region. The nonvaccinated group maintained subthreshold and stable P/N values throughout the monitoring period (Fig. 1).

The negative control grouse survived to the end-point of the test and maintained normal behavior and weight; it is not

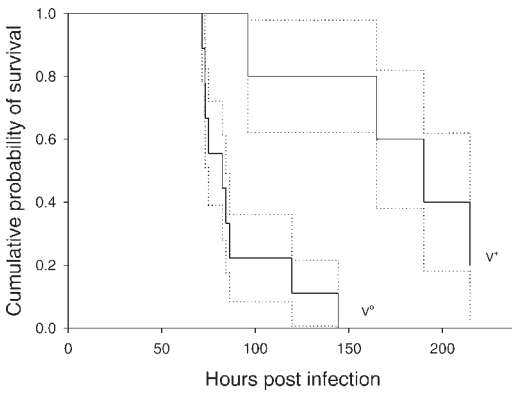


FIGURE 2. Kaplan-Meier survival estimates (solid lines) for vaccinated (V^+) and nonvaccinated (V^-) greater sage-grouse challenged with WNV. Dotted lines depict $\pm 95\%$ confidence limits.

specifically referred to in the remainder of the study.

WNV infection caused 100% mortality in nonvaccinated grouse (Fig. 2). Vaccinated birds survived longer than the nonvaccinated birds ($F_{18,8}=4.710$, $P=0.016$). The mean Kaplan-Meier estimate for 50% survival was 9.5 days for the vaccinated group and 4.5 days for the nonvaccinated group. The actual mean survival time of experimental subjects was 3.7 ± 0.3 days for the nonvaccinated grouse and 6.7 ± 1.1 days for the vaccinated birds that died. One vaccinated bird cleared the virus from its blood but died a day later. Another vaccinated bird cleared the virus from its blood and survived to the end-point of the experiment (14 days). This grouse never showed any overt signs of illness. The control grouse survived to the end-point of the experiment.

Grouse that died of WNV infection all showed similar overt signs of illness, regardless of vaccination status. The first signs of illness were noted during blood sample collection. Some birds had a profuse, clear, watery, oral and nasal discharge. Shortly thereafter, the same birds were observed to piloerect their feathers, shiver, isolate themselves from the group, remain immobile, and seek out walls or other structures to rest against. Within

hours, drooped wings, ataxia, copious oral and nasal secretions, and labored breathing were apparent. If forced to move, the grouse would stumble and right themselves with difficulty. During the final stages of illness, birds would no longer attempt to escape or were incapable of coordinated locomotion. At this point some birds died during sample collection; the remaining birds were euthanized as they were deemed to have reached the end-point of illness. From first overt signs of illness to the end-point took less than 6 hr.

Peak viremia occurred 3 days post-infection for the nonvaccinated group of grouse (Table 1, Fig. 3). This coincided with 80% of the mortality of the nonvaccinated group. Onset of oral and cloacal shedding of viral material lagged 1 day behind initial detection of virus in serum. The quantities of viral material collected from swabs were higher in the oral secretions relative to the cloacal samples. The peak viremia for the vaccinated group was substantially lower relative to the nonvaccinated group (Fig. 3). In addition, the viremia profile was shifted 1 day later. The pattern of oral and cloacal shedding in the vaccinated grouse was similar to that seen in the nonvaccinated group (Table 1). In the 2 longest surviving grouse, oral and cloacal shedding persisted (7–14 days) even after the viral material was cleared below detection limits in the serum (7 days).

Flavivirus-specific IgM antibody was not detected until the sixth day post-infection in any of the grouse (Fig. 4a). Thus, in most of the nonvaccinated grouse, no detectable flavivirus-specific IgM antibody was present before their death. The single grouse in the nonvaccinated group that survived to 7 days postinfection did develop flavivirus-specific IgM antibody. For the vaccinated group an increasing titer for flavivirus-specific IgM antibody was observed from 6 to 9 days postinfection. After 9 days, the titer was stable.

TABLE 1. Concentration of West Nile virus in specimens as a function of vaccination status and time since infection.

Day	N ^b	Nonvaccinated group									N	Vaccinated group								
		Serum ^a			Oral ^a			Cloacal ^a				Serum			Oral			Cloacal		
		n ^c	X ^d	±SE	n	X	±SE	n	X	±SE		n	X	±SE	n	X	±SE	n	X	±SE
0	9	9	nd	—	0	nd	—	0	—	—	5	0	nd	—	0	nd	—	0	nd	—
1	9	9	1.7	0.2	0	nd	—	0	—	—	5	4	−0.1	0.6	0	nd	—	0	nd	—
2	9	9	5.4	0.3	9	1.1	0.3	9	2.0	0.3	5	5	0.0	0.6	1	−1.0	0.0	0	nd	—
3	8	8	6.4	0.2	8	3.1	0.3	8	2.9	0.3	5	5	1.3	0.9	5	0.1	0.5	2	0.3	0.4
4	2	2	5.9	0.2	2	3.1	0.7	2	2.2	0.4	5	5	2.3	0.6	5	1.2	0.8	4	0.3	0.7
5	1	1	6.1	0.0	1	4.8	—	1	3.8	—	4	4	1.2	0.4	4	1.9	0.5	3	0.9	0.9
6	1	1	6.1	0.0	1	5.4	—	1	3.8	—	4	4	1.2	0.2	3	1.7	1.1	3	0.4	0.5
7											4	4	0.9	0.2	4	1.8	1.1	4	0.3	0.2
8											2	1	−0.3	0.0	2	1.6	1.2	2	−0.1	1.1
9											2	0	nd	—	2	0.9	0.9	1	−0.4	0.0
10											1	0	nd	—	1	0.2	0.0	1	−0.3	0.0
11											1	0	nd	—	1	0.3	0.0	1	0.4	0.0
12											1	0	nd	—	0	nd	—	0	nd	—
13											1	0	nd	—	1	−0.4	0.0	1	−0.7	0.0
14											1	0	nd	—	1	−0.9	0.0	1	−0.7	0.0

^a Mean (X) censored quantity of West Nile virus detected in serum, oral swabs, and cloacal swabs±standard error (SE), expressed as Log₁₀(PFUs/ml).

^b N is the number of birds tested. Sample sizes less than 9 for the nonvaccinated group and 5 for the vaccinated group reflect mortality.

^c n is the number of birds with detectable virus. Assay detection limit was 0.1 PFUs/ml, where PFU is plaque-forming unit. Values below threshold are listed as “nd” in the table.

^d $X = \sum PFUs_i / n$, where PFUs is the quantity of virus detected in the *i*-th individual.

Flavivirus-specific IgG antibody never rose above threshold in the nonvaccinated grouse (Fig. 4b). Levels of flavivirus-specific IgG antibody in the vaccinated group were slightly elevated at the onset of the experimental infection relative to negative control serum (Fig. 1) but did not rise above threshold until 9 days postinfection. By this time only 2 of the 5 birds were alive in the vaccinated group. The bELISA appeared to be less sensitive than the capture enzyme-linked immunosorbant assay (cELISA) for IgM (Fig. 4a, c). Values above the preinfection baseline appeared to lag the cELISA by 2 days. This lag most likely represents detection of WNV-specific IgM antibody by the bELISA because the iELISA did not reach suprathreshold value until 9 days postinfection (Fig. 4b, c).

DISCUSSION

Greater sage-grouse are highly susceptible to West Nile virus (WNV) infection. These experimental data not only confirm field studies documenting the susceptibility of greater sage-grouse to WNV (Naugle et al., 2004), but emphasize the rapidity with which the virus affects grouse. It appears that once a sage-grouse becomes infected there is little to no chance of surviving. With the spread of WNV into sage-grouse habitat throughout the western USA, this observation does not bode well for the long-term conservation of this species.

Viremia developed at a similar pace in greater sage-grouse relative to bird species deemed to be highly susceptible to WNV infection, for example, corvids (Fig. 5;

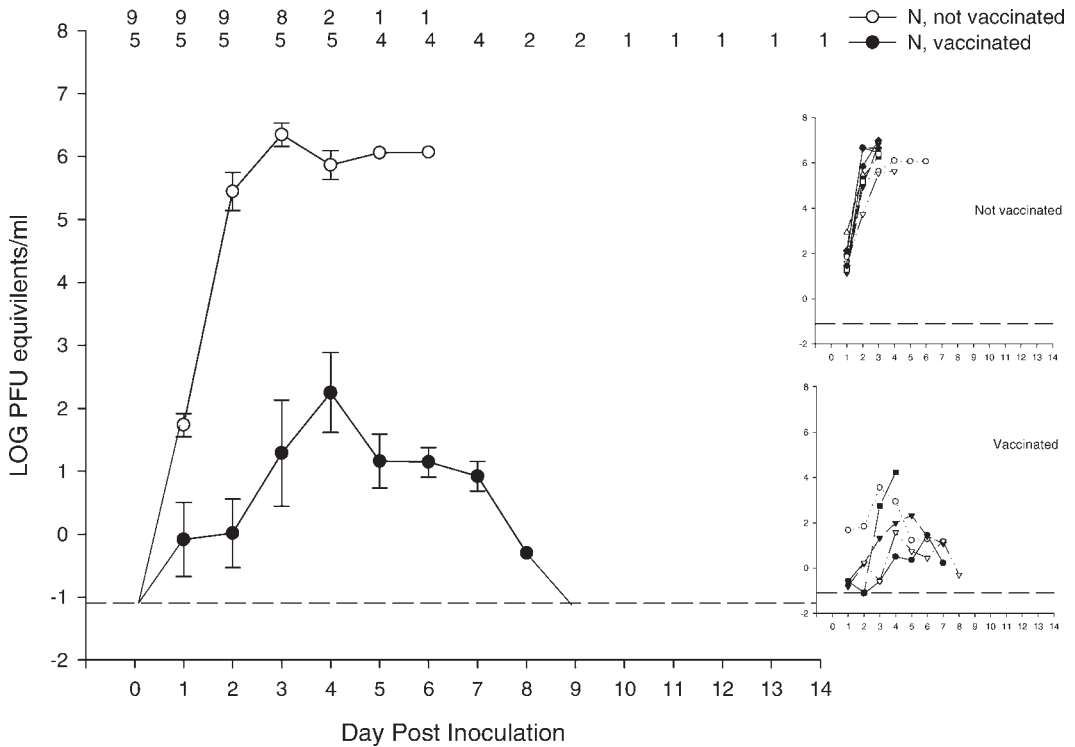


FIGURE 3. Mean (\pm SEM) viremia, as indicated by RT-PCR, as a function of time and vaccination status for greater sage-grouse challenged with WNV. Dashed line depicts the detection limit ($10^{-1.1}$ PFUs/ml) for the RT-PCR method. Numerical insets indicate the number of grouse alive and sampled for RT-PCR analysis of serum. Graphical insets depict individual viremia profiles for vaccinated and nonvaccinated groups.

Turell et al., 2003; Komar et al., 2003; Weingartl et al., 2004). Peak viremia for sage-grouse was well within the median range for peak viremia reported for other species (Fig. 5). Although data on mortality rates for other species of birds are few, greater sage-grouse should be considered a highly susceptible species among birds since all greater sage-grouse died after being experimentally infected with WNV. Of the 25 species of birds experimentally infected by Komar et al. (2003), 8 showed mortality, and 4 of these showed 100% mortality: American crow (*Corvus brachyrhynchos*), black-billed magpie (*Pica hudsonia*), ring-billed gull (*Larus delawarensis*), and house finch (*Carpodacus mexicanus*). Mortality was high for other species as well (33–75%). For the most susceptible species, the mean time to mortality was 6.8 ± 0.8 days until death

(Komar et al., 2003). The mean time until death for greater sage-grouse was 3.7 ± 0.3 days.

Preliminary histological studies indicate that the virus was located throughout the major organs (Cynthia Smeraski and Larry Clark, unpubl.). Gross necropsy showed no overt signs of organ damage. Given the copious oral and nasal secretions and labored breathing, it appears that the cause of death may be related to congestive heart failure or pulmonary edema.

Although the number of greater sage-grouse available for the vaccination studies was initially limited and the success, in terms of survivorship, was low, there is room for optimism. Two vaccinated birds cleared the virus from their blood, and 1 bird survived to the end-point of the experiment. This bird was apparently in good condition, having normal behavior

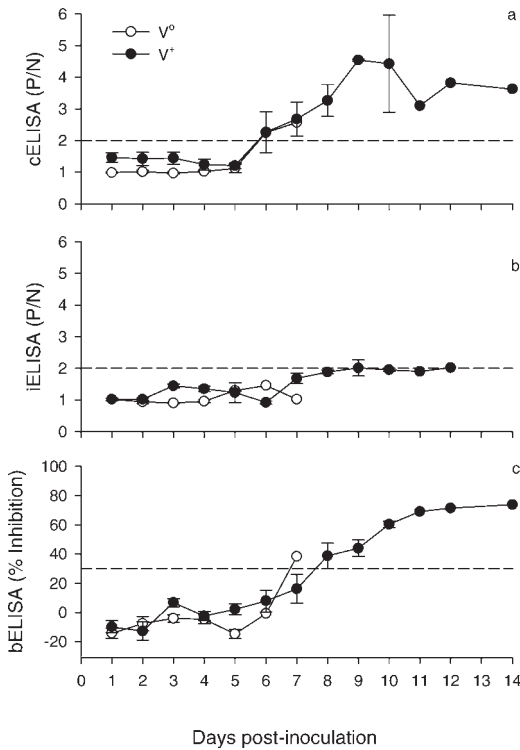


FIGURE 4. Mean (\pm SEM) antibody to WNV in greater sage-grouse serum as a function of ELISA assay and time. (a) cELISA detects IgM, (b) iELISA detects IgG, and (c) bELISA detects IgM and IgG antibodies to WNV. Horizontal dashed lines depict the threshold value for each assay.

and weight. Vaccinated grouse had lower viremia relative to nonvaccinated grouse, and the peak viremia for the vaccinated group was delayed by 1 day. Together these patterns indicate that the vaccine was successful in immunizing the birds, allowing them to clear the virus from the blood. However, because of persistent cloacal and oral shedding (Table 1) and initial immunohistochemical analysis indicating pervasive infection of tissue, it appears that the DNA vaccine was only partially effective at clearing the virus from all tissue.

Grouse in the nonvaccinated group died before an antibody response was detected. Vaccinated grouse seroconverted for flavivirus immunoglobulin G (IgG). This was a precondition for the subsequent experimental inoculations. However, the posi-

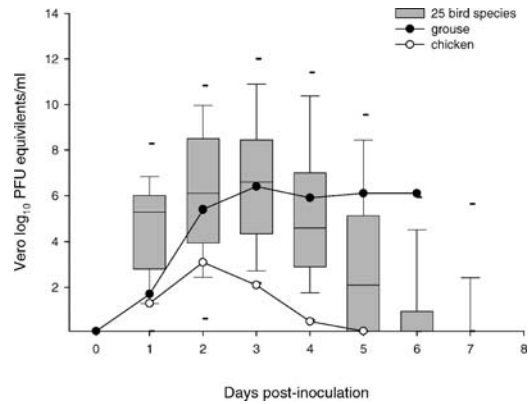


FIGURE 5. Comparison of viremia profiles for greater sage-grouse (solid circles), chickens (open circles), and 25 other species of birds (bars) challenged with WNV. Boxes encompass the 25th and 75th percentiles of mean values reported by Komar et al. (2003). Capped vertical lines encompass the 10th and 90th percentiles of viremia, and dashes encompass the 5th and 95th percentiles of viremia values reported for the 25 species of birds. Viremia profile for the chicken was adapted from Langevin et al. (2001).

tive to negative ratio (P/N) (indirect ELISA [iELISA]) was lower than observed in the high-titer positive control chicken serum, suggesting that improvements could be made in stimulating the antibody response. Nonetheless, even the relatively weak antibody response to vaccination apparently was sufficient to allow grouse to survive to the point where further antibody response was apparent after WNV challenge. Therefore, grouse in the vaccinated group were better able to clear the virus from their blood and survived longer relative to the nonvaccinated group. Thus, the vaccine approach shows promise as a conservation tool, and several possibilities exist to increase the efficacy of the vaccinations. Increased immunoprotection may be gained by adding the NS1 gene of WNV to the fowl pox. NS1 has been shown to be an important immunogen in other systems and could be added to the vaccine construct relatively easily (Hall et al., 2003). The goal would be not only to clear virus from the blood, but also to decrease

the viral load in other tissue. Increasing the efficacy of the vaccine may be possible by using different adjuvants, diluents, or vaccination regimes as well.

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