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Abstract: An epizootic of eastern equine encephalitis (EEE) virus infection in 1984 resulted in death for 7 of 39 captive whooping cranes (*Grus americana*) at the Patuxent Wildlife Research Center. This represented the first known whooping crane deaths associated with this arboviral disease and posed a serious risk to the continued propagation of this endangered species. Subsequent research and surveillance procedures initiated to prevent EEE viral infections in captive whooping cranes included vector surveillance and control, virus surveillance through use of sentinel birds, immunoassays for rapid detection of EEE virus antigen in bird sera and in mosquitoes, and testing of an EEE virus vaccine in whooping cranes. Based on results of these efforts, we are optimistic that EEE virus can be effectively monitored and prevented and thus the risk of future infections can be reduced among captive whooping cranes.

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An outbreak of eastern equine encephalitis (EEE) virus at the Patuxent Wildlife Research Center (Center), U. S. Fish and Wildlife Service, Laurel, Maryland, USA during the late summer and fall of 1984 resulted in death of 7 of 39 endangered whooping cranes (Dein et al. 1986; Carpenter et al. 1987). Viral assays of tissues from 5 of the cranes yielded EEE virus. Epizootiological observations following the outbreak revealed that 14 (44%) of the 32 surviving whooping cranes and 13 (34%) of 38 co-resident sandhill cranes (*G. canadensis*) had EEE virus neutralizing (N) antibody (Clark et al. 1987). No clinical signs were observed in the surviving 32 whooping cranes, and no mortality or clinical signs were observed in 248 sandhill cranes located near the whooping cranes. EEE virus has

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been responsible for extensive mortality in several avian species including the chukar (*Alectoris chukar*) (Moulthrop & Gordy 1960), house sparrow (*Passer domesticus*) (Byrne et al. 1961), and ring-necked pheasant (*Phasianus colchicus*) (Sussman et al. 1958), all exotic species introduced into the U.S. However, the whooping crane losses represent the first reported deaths associated with a natural EEE viral infection in an avian species indigenous to North America.

The EEE virus is enzootic in the eastern and northcentral United States and adjacent Canada, in scattered areas of Central and South America, and in the Caribbean region (Monath 1979). The enzootic cycle involves transmission of virus between wild birds by mosquitoes, with occasional epizootics in equines. *Culiseta melanura* is considered the primary enzootic vector of EEE virus in North America (Hayes 1961; Williams et al. 1972). *Culiseta melanura* breeds in heavily-shaded seepage areas (i.e. in root holes under fallen trees) associated with freshwater swamps. Other aspects of the ecology of *C. melanura* have been previously detailed (Joseph & Bickley 1969). Immediately after the epizootic in the whooping cranes, EEE virus N antibody was detected in wild birds captured at the Center. Serum from one whooping crane and two sandhill cranes bled at the Center in 1974 also had N antibody to EEE virus. During earlier studies at the Center, EEE virus antibody also was found in 5 species of wild birds (Herman 1962). These findings suggested that EEE virus was enzootic and thus posed a serious risk to the future successful propagation of the whooping crane at the Center (Clark et al. 1987). To minimize this risk, a multifaceted strategy was initiated to prevent future EEE viral infection in the captive cranes. This multiagency effort included vector surveillance and control, serological surveillance with sentinel northern bobwhite (*Colinus virginianus*), virological surveillance in *C. melanura*, and a program for vaccination of cranes with an EEE viral vaccine. This paper reports on the research progress and monitoring procedures implemented at the Center to prevent future EEE viral infections in captive whooping cranes.

METHODS AND MATERIALS

Vector Surveillance

Aerial and ground surveys were conducted in March and April 1985 at the Center, Fort George G. Meade (FGGM), and adjacent areas to locate potential overwintering larvae at *C. melanura* production sites. During the spring, summer and fall from 1985-1987, identified production sites were monitored and surveys were conducted to locate additional sites.

Adult *C. melanura* abundance was monitored using standard CDC light traps augmented with a $C0_2$ attractant (dry ice) from May through November 1985-1987. Light traps were placed in two areas within the Center's crane propagation area and along the 7.3 km common boundary between the Center and FGGM, an area with potential breeding and overwintering habitat for *C. melanura*. Eleven trap sites were established at FGGM in 1985, 4 of which also were used in 1986 and 1987, and were checked 2 nights per week. Five to 7 traps were operated 1 night per week in all 3 years at the Center. Additionally, up to 3 standard New Jersey light traps and up to 19 0.28 m^2 resting boxes (see Edman et al. 1968) were used intermittently for supplemental surveillance at the Center.

Vector Control

In 1985, 5 sites positive for *C. melanura* larvae and 35 other potentially positive sites were treated with Altosid[a] briquets (7.9% methoprene). Thirteen days after application of this insect growth regulator, larvae were collected for post-treatment evaluation. Flit MLO[b] (98.5% mineral oil) was also applied to these larva-positive sites. Post-treatment larval behavior was observed and recorded as a qualitative indicator of treatment effectiveness.

Control of adult *C. melanura* was attempted in August 1985, on a 4-hectare plot at FGGM using Dursban 4E[c] (41.2% chlorpyrifos) at a rate of 120 ml per acre and delivered with a Bean Model 100K[d] mistblower. Change in pre- and post-treatment collections in CDC light traps were used as an indication of insecticide treatment efficacy.

[a] *Zoecon Corporation, Palo Alto, California. Use of trade names does not imply endorsement by U.S. Government agency.*
[b] *Exxon Company, Houston, Texas.*
[c] *Dow Chemical Company, Midland, Texas.*
[d] *FMC Corporation, Jonesboro, Arkansas.*

Serological Surveillance

To monitor EEE virus activity during the summer and fall of 1985, 1986, and 1987, 5 seronegative adult northern bobwhites were retained in each of 4 wire mesh cages positioned along the northern perimeter of the Center adjacent to FGGM. The sentinel bobwhites were bled weekly, biweekly, or monthly and the serum component assayed by either the plaque reduction neutralization test (PRNT) for antibody against EEE virus (Clark et al. 1987) or the tissue culture neutralization test. All sera were heat treated and screened for antibody at 1:10 by the PRNT and at 1:4 by the tissue culture neutralization test.

Viral Surveillance

A viral surveillance program was conducted to isolate and identify EEE virus from mosquitoes and crane sera to monitor transmission activity by mosquitoes and to provide corroborative information for diagnosis of crane infections. During these analyses, a traditional cell culture bioassay was compared with a newly developed antigen detected enzyme immunoassay (EIA). In 1985, the EIA for detecting EEE virus in *C. melanura* was initiated with mosquitoes collected from the Center and FGGM. In addition, 86 whooping and sandhill crane serum samples, including 2 from whooping cranes that died in the 1984 epizootic, were analyzed using the EIA. Laboratory procedures for the EIA have been described by Scott et al. (1987).

Vaccination Program

Although a vaccination program was considered feasible, existing information on EEE vaccines was derived primarily from studies of human subjects (Maire et al. 1970). Data indicated that the vaccine was safe for use in humans, and that neutralizing antibody was elicited, but boosters were required to sustain detectable antibody. While limited vaccine studies have been attempted for prevention of EEE in pheasants, results were inconclusive and based only on a single vaccination (Sussman et al. 1958; Snoeyenbos et al. 1978; Eisner & Nusbaum 1983). Therefore, in order to determine the effect of administering a formalin-inactivated EEE vaccine to whooping cranes, sandhill cranes were used in preliminary studies to assess safety and to determine magnitude and duration of antibody following different routes of vaccination. Three routes of inoculation (intramuscular [IM] subcutaneous, and intravenous), safety of the vaccine, and various dosage regimens were tested (Clark et al. 1987). All serum samples were assayed for EEE virus N antibody using the PRNT in Vero (African green monkey kidney) cells (Clark et al. 1986). An 80% or more reduction of the virus dose by a specific dilution of crane serum was considered evidence of EEE virus N antibody.

In 1985, all (n = 15) EEE viral N antibody-seronegative whooping cranes at the Center and 4 whooping cranes with pre-existing, naturally acquired N antibody received an IM injection of 0.5 ml of vaccine followed by a 1.0 ml booster IM 30 days later (Clark et al. 1987). After 6 months, these whooping cranes were rebled and given 1.0 ml of vaccine IM. In July 1986 and 1987, all whooping cranes over one year of age were bled via jugular venipuncture and administered 1.0 ml of EEE virus vaccine IM. These birds were rebled 30 days post vaccination, and those with titers <1:10 were revaccinated. Young-of-the-year were administered 0.5 ml and 1.0 ml vaccine approximately 30 days apart beginning in August or September. All serum samples were evaluated for hemagglutination-inhibition (HI) antibody against EEE virus and/or were assayed for EEE virus N antibody using the PRNT (Clark et al. 1987).

RESULTS

Vector Surveillance

The initial aerial space survey of the Center and FGGM identified habitat at FGGM which appeared suitable for overwintering *C. melanura* within a 4 km radius of the Center's crane propagation area. Subsequently, ground surveys confirmed the presence of fourth instar *C. melanura* larvae in 5 (11%) of 44 sites sampled. Larval numbers ranged from 1 to greater than 100 per site. Periodic larval sampling was performed within a 5.6 km radius of the cranes through 1987, but no new *C. melanura* larva-positive sites were found.

From 1985 to 1987, 122,035 mosquitoes were collected at the Center and FGGM, with 73,350, 18,175 and 30,510 mosquitoes collected in 1985, 1986 and 1987, respectively (Table 1). During the 3 years of vector surveillance, *C. melanura* comprised 2.3% of all mosquitoes collected (Table 1). *Culiseta melanura* comprised 2.3% of the species composition in 1985, 3.1% in 1986J and 1.9% in 1987. Female *C. melanura* were collected from mid-May through late October with the greatest num-

ber found from late August through early September. At the collection sites in the Center's crane propagation area, *C. melanura* was rarely collected. *Coquillettidia perturbans* comprised 20.7% to 41.5% of the annual collections and was the single most abundant species.

Vector Control

Because vector control activities were only conducted on a limited scale, evaluations of the control efforts were considered preliminary. All larvae from a sample of fourth instar *C. melanura* (n = 18) collected from a site treated with methoprene failed to develop past the pupal stage. Mineral oil appeared to effect *C. melanura* larval respiration in treated sites, although the surface oil film did not last for more than 2 or 3 days as a result of precipitation and wind. An approximately 4-fold increase between the pre-and post-treatment adult *C. melanura* trap index at one treatment site indicated that a single application of chlorpyrifos for adult control did not reduce the C. melanura population to below the pre-treatment level.

Serologic Surveillance

All sentinel bobwhite sera obtained in 1985, 1986 (August to October) and 1987 were negative for EEE virus antibody.

Viral Surveillance

A total of 910 adult female *C. melanura* were collected at the Center and FGGM during 1985 and assayed for EEE virus in 133 pools. No EEE virus was isolated in 2 different cell culture bioassay systems or by the EIA. Results from an evaluation of the EIA using mosquitoes collected elsewhere in Maryland indicate that the test was effective; there were no false positives or false negative results (Scott et al. 1987). In addition, the same EIA procedure correctly identified EEE virus antigen from 2 whooping crane tissue specimens that contained infectious virus (Scott & Olson 1986). Both cranes had died during the 1984 epizootics.

Vaccination Program

The formalin-inactivated EEE vaccine produced antibody in cranes following IM vaccinations, whereas subcutaneous and intravenous routes failed to elicit a response (Clark et al. 1987). Adverse reactions to the vaccine were not observed in the IM or subcutaneous vaccinated cranes. However, the intravenous inoculated cranes developed various degrees of temporary ataxia within 15 minutes.

Among the 32 whooping cranes that survived the EEE epizootic in 1984, 14 had N antibody to EEE virus, thus indicating that these cranes were naturally infected either during or prior to the epizootic. The antibody response for 4 following vaccination with the EEE vaccine was characterized by a rapid and, in general, sustained high antibody titers that differed from the low transitory pattern exhibited by the seronegative cranes (Clark et al. 1987). This demonstrated that the vaccine was immunogenic and that the antibody detected following vaccination of seronegative cranes was elicited by the EEE vaccine, rather than possible exposure to a natural EEE viral infection.

Because it was determined that a primary and booster vaccination were necessary to sustain detectable antibody, this regime initially was used in 15 seronegative whooping cranes. Only 1 of 12 cranes tested following primary vaccination developed detectable antibody, whereas 10 of the other 11 cranes had antibodies 60 days following administration of a booster on day 30. Thereafter, antibody titers waned, such that only 2 of 14 cranes had detectable antibody on day 180 post-vaccination. After a second booster on day 180, all cranes tested(n=13) had detectable antibody with a geometric mean titer of 1:160 on day 210, or a 4-fold or greater titer as compared to those observed on day 30 or 60 after the primary inoculation and booster. All cranes were still positive on day 255, but titers were 4-fold lower, and by day 470 only 3 of 14 cranes had detectable antibody. A third (i.e., the annual) booster given on day 470 resulted in the production of detectable antibody in 7 of 12 cranes at day 500 and in 14 of 14 cranes on days 560 and 817. The geometric mean titer for the last 2 dates was 1:80 and 1:40, respectively.

DISCUSSION

Results of larval surveys indicated that there were *C. melanura* at FGGM within 4 km of the Center's crane propagation area. This population could have been instrumental in causing the 1984 EEE epizootic. However, adult *C. melanura* populations in the study area, during 3 years of post-outbreak surveillance, were low compared to population levels observed on Maryland's Eastern Shore, an area where EEE virus is endemic (Joseph & Bickley 1969; Scott et al. 1987). The large num-

ber of *C. melanura* present (34.2% of all mosquitos collected) on the study area merits attention and further evaluation, since this species has been reported to be an important epidemic vector for EEE virus (Engemann 1982). Larval control (Hayes 1962) may have merit in managing *C. melanura* populations at the Center and FGGM because of the limited number of production sites and the environmental concerns associated with adulticides. Adulticiding may be of some value during emergency conditions, but multiple treatments of broader areas would be needed and vehicle access to areas near production sites would be difficult. Chemical adulticiding should only be considered if early viral activity is detected through sentinel bird surveillance, large vector numbers are detected through adult mosquito surveillance, and larval control proves ineffective in suppressing *C. melanura* populations.

No seroconversions occurred in the bobwhite sentinels at the Center from 1985-1987. Because bobwhite have been effective sentinels for monitoring EEE virus transmission in other studies (Williams et al. 1972), sentinel bobwhites will continue to be used in EEE virus management activities at the Center.

The EIA technique was successful in rapidly detecting EEE viral antigen in crane sera. Although no evidence of virus activity was detected in *C. melanura* collected at the Center in 1985, virus isolation and identification results were in complete accord with more laborious and time-consuming cell culture assays systems (i.e. positives were correctly identified and there were no false positives or false negatives). The results indicate the antigen detected EIA is a simple, sensitive and specific alternative to traditional bioassays for EEE virus isolation (Hildreth & Beaty 1984; Hildreth et al. 1984; Scott & Olson 1986; Hildreth & Beaty 1987; Scott et al. 1987; Scott et al. in press). The technique is a valuable addition to the procedures currently available for detection of EEE virus activity near the whooping crane flock.

Data generated in the vaccination studies revealed that a formalin-inactivated human EEE viral vaccine elicited N antibody in both sandhill and whooping cranes (Clark et al. 1987). Whether whooping cranes are protected against natural infection has not been ascertained, but the results indicated that EEE viral N antibody titers induced by the vaccine are indicative of a protective state, and therefore should minimize the risk of captive cranes to EEE viral infection. The vaccine is readily available, inexpensive, and has not caused any apparent adverse effects in cranes when administered IM. In addition, the program can be operated with limited resources, as antibody can be elicited in the cranes by a primary inoculation followed by a 30 day booster and can be sustained by only 1 or 2 annual boosters.

The epizootic of EEE virus in captive whooping cranes represented the most significant loss of captive whooping cranes ever experienced and resulted in recognition of a potentially significant risk to the successful recovery of this species. However, through continuation of the (1) mosquito surveillance and serological surveillance programs, (2) use of the rapid immunoassay, and (3) annual use of the EEE virus vaccine, a program to monitor EEE virus activity and to prevent future EEE viral infections in this endangered species can be successful. Depending on mosquito and viral surveillance results, mosquito control procedures may be useful adjuncts to the aforementioned prevention strategies.

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Table 1. Mosquitoes collected during the EEE viral surveillance program at the Patuxent Wildlife Research Center and Fort George G. Meade, Maryland.

	Year				
Species	1985	1986	1987	Total	(%)
Aedes spp.	4,124	878	2,771	7,773	(6.4)
Anopheles bradleyi-crucians	4,899	1,979	2,698	9,576	(7.8)
Anopheles punctipennis	2,856	1,550	1,749	6,155	(5.0)
Anopheles quadrimaculatus	2,659	372	926	3,957	(3.2)
Coquillatidia perturbans	30,404	5,087	6,305	41,796	(34.2)
Culex erraticus	11,830	3,543	5,270	20,643	(16.9)
Culex salinarius	2,925	1,970	3,341	8,236	(6.7)
Other *Culex* spp.	2,791	1,465	4,494	8,750	(7.2)
Culiseta melanura	1,653	563	583	2,799	(2.3)
Other species or mosquitoes not identified to species	9,209	768	2,373	12,350	(10.1)
Total	73,350	18,175	30,510	122,035	