

EXPERIMENTAL INFECTION OF WADING BIRDS WITH EASTERN EQUINE ENCEPHALITIS VIRUS

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ABSTRACT: To study the susceptibility of wading birds to eastern equine encephalitis (EEE) virus and to determine their potential as reservoir or amplifying hosts, fledgling glossy ibises (*Plegadis falcinellus*) and snowy egrets (*Egretta thula*) were captured in New Jersey (USA) and shipped to Colorado (USA) where they were experimentally inoculated with EEE virus. All 16 snowy egrets and 14 (93%) of 15 of the glossy ibises inoculated became viremic with moderate titers, and all survivors developed neutralizing antibody. Six ibises and two egrets died during the first week after inoculation, and EEE virus was isolated from the tissues of three birds. Our experimental results support field evidence about the relative involvement of glossy ibises and snowy egrets in the epizootiology of EEE virus in New Jersey.

Key words: Arbovirus, eastern equine encephalitis virus, wading birds, experimental infection, reservoir competence, glossy ibis, *Plegadis falcinellus*, snowy egret, *Egretta thula*.

INTRODUCTION

Eastern equine encephalitis (EEE) virus (*Alphavirus*, Family *Togaviridae*) occurs from Canada to Argentina (Acha and Szyfres, 1987) and produces clinical disease in humans; equines and other domestic animal species; exotic game bird species, including recently introduced emus (*Dromaius novaehollandiae*, Tully et al., 1992); and a number of native wild bird species, most notably the whooping crane (*Grus americana*) (Dein et al., 1986). Within the United States, EEE virus first was isolated in the mid-Atlantic coastal region during an epizootic in equines in 1933, and it probably was responsible for epizootics in equines as early as 1831 (Morris, 1989). The virus now occurs in the Atlantic and Gulf coastal states and in isolated foci in some inland states east of the Mississippi River, such as Michigan and New York (USA). Human and equine cases are reported nearly every year within the enzootic areas; 141 human cases were reported during the 29-yr period of 1964 to 1992 (Calisher, 1994).

In the U.S., EEE virus is maintained in enzootic foci in freshwater swamp habitats

in wild bird-mosquito transmission cycles during the summer months in northern latitudes (Dalrymple et al., 1972; Morris et al., 1980; McLean et al., 1985b) and throughout the year in southern latitudes (Stamm et al., 1962; Bigler et al., 1976). Many species of wild birds, mostly passerines, have been incriminated as natural maintenance and amplifying hosts and *Culiseta melanura* has been implicated as the principal mosquito vector in these enzootic foci (Morris, 1989).

Well-established EEE foci have been identified in cedar swamp habitats in southern New Jersey (USA), where the virus is maintained in the usual wild bird-*Cs. melanura* cycle (Crans et al., 1994). The virus generally remains confined to these swamp habitats; however, it occasionally escapes from these foci, probably during the peak of seasonal virus transmission, carried by either infected mosquitoes or viremic birds. Two common species of wading birds, the glossy ibis (*Plegadis falcinellus*) and the snowy egret (*Egretta thula*), roost in wooded swamps and feed in open salt marshes of southern New Jersey. Both species regularly are exposed to EEE virus there, although glossy

ibises have a significantly higher antibody prevalence than do snowy egrets (W. J. Crans, unpubl.). Therefore, these birds could serve to facilitate the movement of EEE virus from enzootic foci if they are susceptible to the virus and are able to infect mosquitoes.

Our objective was to determine the laboratory susceptibility and viremic response of the glossy ibis to infection with EEE virus, to confirm and extend previous investigations on the susceptibility of the snowy egret to EEE virus (Kissling et al., 1954), and to determine the competence and potential of these species as vertebrate reservoir hosts.

MATERIALS AND METHODS

Both glossy ibises and snowy egrets were captured by hand as fledglings at their nests in coastal breeding colonies at Stone Harbor in southern New Jersey (39°02'N, 74°46'W). This area of the state has no known history of EEE virus activity and is virtually free of mosquitoes (W. J. Crans, unpubl.). For shipment, the birds were placed in large animal shipping containers and immediately transported by overnight air shipment to Denver, Colorado (USA), where they were collected and directly transported by vehicle to Fort Collins, Colorado. Because ibises have an earlier breeding season, they were captured and shipped first (24 June 1992); two separate shipments of egrets (15 and 22 July 1992) followed. The birds were examined and marked with numbered leg bands. A blood sample was taken from the brachial vein with a 1 ml syringe and 22-gauge needle; the birds then were placed in wire cages on the floor of a biocontainment level 2 room (Subcommittee on Arbovirus Laboratory Safety, 1980) at the Centers for Disease Control and Prevention laboratory in Fort Collins. Steam-sterilized sand was placed on the floor of each cage; water and food containers were placed inside the cages on top of the sand. A roosting bar was attached within each cage. Birds were fed their natural food; glossy ibises first were fed horseshoe crab (*Limulus polyphemus*) eggs which were collected in and shipped from New Jersey, but they gradually were switched to commercial, canned cat food containing fish or shrimp products provided twice each day. Snowy egrets were maintained on live flathead minnows (*Pimephales promelas*) obtained from a local commercial source and placed in fresh water containers twice each day.

Each species was divided into three separate groups of five birds each, with the exception of one group of egrets which comprised six birds. The birds in each group were inoculated subcutaneously with 0.1 ml of particular dosages of EEE virus (strain NJO/60, World Health Organization Center for Reference and Research, Centers for Disease Control & Prevention, Fort Collins, Colorado): 2.9 log₁₀ plaque-forming units (PFU), 3.9 log₁₀ PFU, and 5.0 log₁₀ PFU of virus, respectively. There were no uninoculated control birds because of the multiple experimental groups used and because preinoculation blood samples from all of the birds were tested for virus and specific antibody. The birds in each treatment group were kept together in separate cages. Blood samples (0.5 ml) were taken as described, daily for 7 days and then on days 14, 21, and 28 postinoculation (PI); samples were drawn from seven of the snowy egrets at 36 days PI. The blood samples were added to an equal amount of diluent consisting of M199 medium (Gibco, Life Technologies, Inc., Grand Island, New York), antibiotics, 1% bovine albumin (Intergen Company, Purchase, New York), and 20% heat-inactivated fetal bovine serum (Hyclone Laboratories Inc., Logan, Utah, USA). The samples were kept cool on wet ice; the serum was removed after centrifugation at 860 × G for 15 min and stored in labeled vials at -70 C until tested. Birds were observed twice daily for signs of illness. Dead birds, which usually were discovered in the morning, were placed immediately into a sealed bag and kept at -70 C until thawed for the collection of tissue samples. At the conclusion of the experiment, surviving birds were euthanized with CO₂ gas.

For the determination of viremia, 0.1 ml of each diluted serum specimen was placed onto a monolayer of Vero cells (American Type Culture Collection, Rockville, Maryland, USA), grown in six-well plastic plates; the sample was allowed to absorb for 1 hr at 37 C (McLean et al., 1985a). An overlay containing nutrient medium, 1% Noble agar (Difco Laboratories Inc., Detroit, Michigan), and 1:25,000 of neutral red (Gibco) was added to the cultures, which then were incubated at 37 C in 5% CO₂ until plaques were counted, or for 10 days. When serum specimens contained plaques too numerous to count, 10-fold dilutions of the specimens were made, and the dilutions were retested in Vero cell cultures to determine endpoints. About 1-cm³ portions of brain, liver, spleen, and heart tissue were aseptically removed from thawed carcasses, washed three times with sterile, phosphate-buffered saline, and macerated in 2 ml of diluent as described with a mortar and pestle. The mixture was centrifuged at 310 × G for 10 min, and 10-fold dilutions were made from each su-

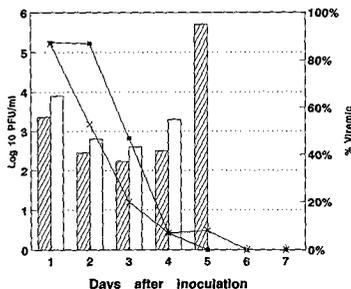


FIGURE 1. Daily mean viremic responses of glossy ibises and snowy egrets inoculated with eastern equine encephalitis virus (NJO/60 strain). Virus titers are expressed as \log_{10} plaque-forming units (PFU)/ml of blood in Vero cell culture. Bars represent virus titers and lines represent the percentage viremic. Bars with diagonal lines and lines with crosses represent glossy ibises; bars with dots and lines with square boxes represent snowy egrets.

pernatant. An 0.1-ml aliquot of each dilution was inoculated onto monolayers of Vero cells for isolation of virus and for determination of virus titers as described for serum specimens.

Serum specimens were heat-inactivated at 56 C for 30 min and tested for neutralizing (SN) antibody against EEE (NJO/60 strain) virus by the constant virus, serum-dilution neutralization test in Vero cell culture grown in six-well plastic plates (McLean et al., 1993). Equal volumes of serum and virus, diluted to contain 100 to 200 PFU, were mixed and incubated overnight at 4 C. Then 0.1 ml of the mixture was added to Vero cell cultures and allowed to absorb for 1 hr at 37 C. The inoculated cultures were overlaid and incubated as described until plaques were counted. A reduction in PFU by 80% or more compared with positive and negative control serum specimens was considered positive. Serum specimens positive for SN antibody were diluted two-fold and retested to confirm the results and determine antibody titers. The data were analyzed with the Chi-square and Student's *t*-tests (Sokal and Rohlf, 1981).

RESULTS

With a single exception, all birds survived their capture and shipment to Colorado and readily adapted to the laboratory situation; one glossy ibis failed to eat in captivity and died of malnutrition. No

viruses were isolated from bloods taken from glossy ibises or snowy egrets before the start of the experiment, and none had detectable SN antibody against EEE virus.

All 16 snowy egrets and 14 (93%) of 15 glossy ibises became viremic following inoculation with EEE virus. No significant differences were found in the viremic responses of either species to the three different dosages of EEE virus inoculated; therefore, the results were combined for further analyses. The mean (\pm SE) peak viremia titer ($3.83 \pm 0.42 \log_{10}$ PFU/ml for egrets and 3.43 ± 0.35 for ibises), mean overall viremia titer (3.2 ± 0.23 and 3.0 ± 0.21 , respectively), and mean duration of viremia (2.2 ± 0.16 days and 1.9 ± 0.29 days, respectively) were all slightly but not significantly higher in the snowy egrets (Table 1). There were variable responses for both glossy ibises and snowy egrets (Fig. 1); most birds had low viremia titers of 1 or 2 days' duration, while others had moderate to high viremia titers of 3 or 4 days duration. Two ibises had viremia patterns that were quite different from each other (Fig. 2), and both died by day 6 PI; EEE virus was isolated from the tissues of only one of these birds (Bird 1). One snowy egret had an unusually high viremia titer ($10^{6.3}$ PFU/ml) of 1 day's duration; it died the next day.

Six ibises and two egrets died during the experiment; one apparently well egret died during handling on day 3 PI. Generally, sick birds first stopped eating and became lethargic; some had drooping wings and ataxia, and all died 1 to 3 days after these behavioral changes first were noted. One snowy egret that did not die had a bloody discharge from its mouth, starting on day 5 PI and lasting for 4 days. Virus was isolated from tissues from one of the six glossy ibises and from both snowy egrets that died (Table 2). Eastern equine encephalitis virus was isolated from heart tissue of these three birds and only from the heart of the egret that died during handling. Virus also was isolated from the spleen of the ibis and from the brain, liver, and spleen of the

TABLE 1. Response of three species of water birds to inoculation with eastern equine encephalitis virus (strain NJO/60).

Species	Number tested	Number viremic	Viremia (PFU/ml) ^a				Percent antibody-positive
			Mean (\pm SE) peak titer ^b	Range of peak titer	Mean (\pm SE) overall titer	Mean duration (days)	
Glossy ibis	15	14	3.4 \pm 0.35	2.0–6.5	3.0 \pm 0.21	1.9	100
Snowy egret	16	16	3.8 \pm 0.42	2.0–9.3	3.2 \pm 0.23	2.2	100
Whistling ducks ^b	8	6	3.3 \pm 0.27	2.5–4.2	2.7 \pm 0.18	2.1	100
Snowy egret ^c	6	6	4.3 \pm 0.21	3.3–4.8	3.4 \pm 0.32	2.9	100

^a Titers expressed as \log_{10} plaque-forming units (PFU) in Vero cell culture per ml of viremic blood.

^b Results from Aguirre et al. (1992).

^c Results from Kissling et al. (1954), titers expressed as \log_{10} mouse LD₅₀ per 0.03 ml of viremic blood at 24-hr intervals.

second egret. High titers of virus were present in the tissues of this egret (Table 2), which was the bird with the very high viremia titer ($10^{9.3}$ PFU/ml) on the day before its death.

Serum neutralizing antibody began to appear by the end of the first week PI, and all surviving birds of both species had detectable antibody by the third week PI (Fig. 3). Antibody developed faster in glossy ibises (78% were antibody positive vs. 50% of snowy egrets at 2 wk PI), but antibody titers were significantly higher ($T = 3.68$, $P < 0.001$) in snowy egrets up to 4 wk PI (1:116 vs. 1:76).

DISCUSSION

Both glossy ibises and snowy egrets were highly susceptible to inoculation with EEE virus; even the lowest dose of virus used in the study was above the 50% infectious dose (ID_{50}) for these two species. Both species developed similar viremic responses, although these responses were not uniform among individual birds. As used in this study, artificially induced infections in water birds has been shown to yield results similar to those produced by transmission from infected *Aedes aegypti* mosquitoes (Kissling et al., 1954). The viremia titers of the glossy ibises and snowy egrets in this study were considerably lower than pre-

viously observed in some passerine species, but were similar to viremia titers seen previously in snowy egrets and other wading bird species by Kissling et al. (1954) and in whistling ducks (*Dendrocygna autumnalis*) by Aguirre et al. (1992) (Table 1).

Both species developed a moderate SN antibody response (100% positive by 3 wk PI, Fig. 3), in contrast to the rapid response of snowy egrets infected during a previous study (Kissling et al., 1954) in which all were antibody positive by 1 wk PI. The

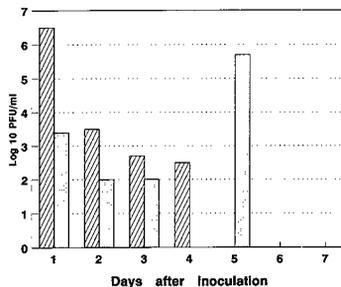


FIGURE 2. Daily viremic responses of two individual glossy ibises inoculated with eastern equine encephalitis virus (NJO/60 strain). Virus titers are expressed as \log_{10} plaque-forming units (PFU)/ml of blood in Vero cell culture.

TABLE 2. Virus isolation from tissues of glossy ibises and snowy egrets that died following inoculation with eastern equine encephalitis virus (strain NJO/60).

Species	Number positive/ number tested	Tissues tested	Virus titer*
Glossy ibis	1/6	Brain	<1.0
		Heart	4.3
		Liver	<1.0
		Spleen	2.7
Snowy egret	2/2	Brain	6.7
		Heart	6.6
		Liver	8.3
		Spleen	2.0
		Blood (previous day)	9.3
		Brain	<1.0
		Heart	2.2
		Liver	<1.0
		Spleen	<1.0

* Virus titer expressed as \log_{10} plaque-forming units of virus in Vero cell culture per ml of tissue.

snowy egrets in this study had significantly higher titers of antibody during the first few weeks, particularly at 3 wk PI, than the glossy ibises. However, the ibises produced equal titers after 3 wk PI. Apparently, antibody titers had not reached their peak then since the titers continued to in-

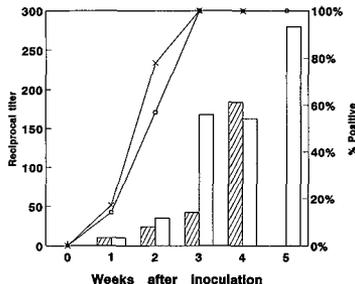


FIGURE 3. Neutralizing antibody response of glossy ibises and snowy egrets inoculated with eastern equine encephalitis virus (NJO/60 strain). Bars represent the mean reciprocal antibody titer as determined in the plaque-reduction neutralization test in Vero cell culture and lines represent the percentage positive. Bars with diagonal lines and lines with crosses represent glossy ibises; bars with dots and lines with square boxes represent snowy egrets.

crease in the one group of seven egrets that were kept for an additional week.

In an associated study, colony-reared *Aedes albopictus* mosquitoes were allowed to feed, and fed readily on selected EEE-inoculated snowy egrets used in this study (Mitchell et al., 1993). These mosquitoes became infected with EEE virus after feeding on viremic egrets; 60% became infected after ingesting $10^{3.1}$ PFU of virus in the bloodmeal from one of the egrets. The ID_{50} for *Ae. albopictus* was calculated to be $10^{2.8}$ PFU, and the minimum threshold of infection was ≤ 10 PFU per bloodmeal. The infection threshold and ID_{50} observed for *Ae. albopictus* (Mitchell et al., 1993) were similar to those parameters determined previously for *Ae. sollicitans* (Sudia et al., 1956). *Aedes sollicitans* is suspected to be an important epizootic and epidemic vector for EEE in southern New Jersey (Crans et al., 1986), and, if given the opportunity, this vector will feed on ciconiform birds (Crans et al., 1990).

Mortality was higher for the glossy ibises than for egrets; however, since no histopathologic examination was performed and since there was no separate control group, death caused by EEE virus could only be assumed in the one egret that had a disseminated, high-titered infection, includ-

ing infection of the central nervous system. Death from EEE infection has been documented in a number of exotic avian species (Morris, 1989; Tully et al., 1992) and in native Icteridae (Kissling et al., 1954) and Gruidae (Dein et al., 1986) species. The extreme differences in the viremic responses of some birds, particularly the snowy egret with the very high titers of disseminated virus, and death in some birds could have resulted from synergistic factors not tested in this study, such as debilitating avian retroviruses.

From these studies, we conclude that glossy ibises and snowy egrets are susceptible hosts for the transmission of EEE virus from the enzootic vector *Cs. melanura* in freshwater swamp habitats and could serve subsequently as a virus source for the epizootic vector *Ae. sollicitans* in salt marsh habitats of New Jersey. However, the glossy ibis appears to be a more important bridging host for the escape of EEE virus from enzootic swamp habitats in southern New Jersey because of its early and long feeding period in the salt marsh areas during the summer season, its relatively high natural exposure to EEE virus (W. J. Crans, unpubl.), and its susceptibility and potential reservoir competence for the virus. In contrast, both species appear to be inefficient amplifying hosts within the swamp habitats because they had only moderate viremia titers and *Cs. melanura* has a much higher infection threshold (Chamberlain et al., 1954) than *Ae. sollicitans*.

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