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EXPERIMENTAL INOCULATION OF HOUSE SPARROWS (*PASSER DOMESTICUS*) WITH BUGGY CREEK VIRUS

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ABSTRACT: We performed experimental inoculations of house sparrows (*Passer domesticus*) with Buggy Creek virus (BCRV), a poorly known alphavirus (*Togaviridae*) vectored primarily by the swallow bug (Hemiptera: Cimicidae: *Oeciacus vicarius*) that is an ectoparasite of the cliff swallow (*Petrochelidon pyrrhonota*) and house sparrow. Viremias were detected by plaque assay in two of six birds on days 1–3 postinoculation; viremia was highest on day 2. Viral RNA was detected by reverse transcriptase-polymerase chain reaction (RT-PCR) in blood of six of 12 birds ranging from day 1 to day 15 postinoculation. Infectious BCRV was detected in nasopharyngeal swab samples from two birds by plaque assay. Three control birds that were housed with viremic individuals showed evidence of BCRV RNA in blood (by RT-PCR), suggesting possible bird-to-bird transmission of this virus. Viral RNA also was detected by RT-PCR in brain and skin tissue of six birds on necropsy at the end of the 16-day experiment. Introduced house sparrows are apparently a competent amplifying host for BCRV, and their presence year-round at cliff swallow colonies may facilitate persistence of the virus locally, especially when cliff swallows abandon a site temporarily. The findings that BCRV can be shed orally, that it persists in bird skin, and that control birds could apparently be infected by conspecifics suggest that this virus may be transmitted from bird to bird in the crowded conditions of many cliff swallow colonies.

Key words: Alphavirus, Buggy Creek virus, cliff swallow, house sparrow, *Passer domesticus*, virus transmission.

INTRODUCTION

Wild birds are a common amplifying host for many arthropod-borne viruses (arboviruses) and have been implicated to varying degrees in the transmission cycles of most viruses that infect them. Being highly mobile, birds can potentially move arboviruses over long distances and introduce them to previously unexposed host populations (Stamm and Newman, 1963; Lord and Calisher, 1970; Calisher et al., 1971; Brown et al., 2007). The likelihood of birds transmitting viruses by infecting naïve vectors depends in large part on how long birds maintain infectious virus in the blood. If this period is short, few uninfected vectors will be exposed, and transmission by birds will be low, a scenario thought to limit the spread of some viruses by birds (Reeves, 1974; Rappole and Hubálek, 2003).

Experimental studies on the alphaviruses (*Togaviridae*) have shown that most birds maintain viremias for relatively brief periods. For eastern (EEEV) and western equine encephalomyelitis (WEEV) viruses, for example, a variety of mostly passerine bird species exhibit sufficiently elevated viremia levels to infect mosquitoes for typically the first 1–2 days after inoculation, but viremias are frequently undetectable beyond 3 days postinoculation (Hardy and Reeves, 1990; Komar et al., 1999; Kramer et al., 2002; Reisen et al., 2003). Interestingly, the introduced, nonnative European starling (*Sturnus vulgaris*) was a more effective amplifying host (with higher levels of viremia) for the alphavirus EEEV in Massachusetts than were several native bird species tested (Komar et al., 1999).

Buggy Creek virus (BCRV) is a poorly known alphavirus found widely in the

western Great Plains of North America (Pfeffer et al., 2006). It is a member of the western equine encephalomyelitis virus complex, a group that also includes Highlands J virus, WEEV, SINV, and Aura virus (Reisen and Monath, 1989; Strauss and Strauss, 1994). Buggy Creek virus, and the closely related Fort Morgan virus (likely a strain of BCRV; Pfeffer et al., 2006), are unusual in being one of the few alphaviruses routinely vectored by insects other than mosquitoes: the typical vector is the cimicid swallow bug (Hemiptera: Cimicidae: *Oeciacus vicarius*; Rush et al., 1980; Scott et al., 1984; Hopla et al., 1993; Brown et al., 2001). The hematophagous swallow bug is an ectoparasite primarily of the colonially nesting cliff swallow (*Petrochelidon pyrrhonota*). Lacking wings, the flightless bugs are confined mostly to swallow nests and nesting substrates, even in winter. Swallow bugs also take blood meals from introduced house sparrows (*Passer domesticus*) that occupy cliff swallow nests in many colonies (C. R. Brown, pers. obs.). Buggy Creek virus has been found in about 25% of bug pools tested across multiple years (Brown et al., 2001; Moore et al., 2007), and because of the close association between bugs and cliff swallows and house sparrows at colony sites, both of these birds are assumed to be vertebrate amplifying hosts for BCRV (Scott et al., 1984). However, nothing is known about the duration or intensity of the viremic response to BCRV in avian hosts, or about the degree to which birds amplify and transmit this virus.

In this study we experimentally inoculated adult house sparrows with BCRV to measure the duration and magnitude of viremia, to assess presence of the virus in avian tissue, and to determine the extent of virus shedding and potential for bird-to-bird transmission. That the house sparrow is an invasive species also provides the opportunity to assess host competence for a recent addition to the swallow bug/cliff swallow ecosystem and the potential role this introduced species may have in the population dynamics of BCRV. House sparrows are nonmigratory residents across much of North America, often usurping nesting cavities (e.g., nest boxes) used by native bird species, and they will appropriate the enclosed, retort-shaped nests of cliff swallows. House sparrows typically have relatively long breeding seasons and may also occupy nesting sites in the winter for roosting (Lowther and Cink, 1992; C. R. Brown, pers. obs.) when cliff swallows are absent from colonies.

MATERIALS AND METHODS

Experimental inoculations

Thirty wild house sparrows, 18 males and 12 females, were captured in mist nets on 1 February 2005 at a feedlot in Greeley, Weld County, Colorado, USA, and transported in large cages to the Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention facility in Fort Collins, Colorado. On arrival, we sampled 200 μL of blood via jugular venipuncture from each bird and assayed the sera from these samples for BCRV-specific antibodies determined via plaque-reduction neutralization tests (PRNTs; Beaty et al., 1995). These PRNTs were carried out on Vero cells using a double 0.5% agarose overlay (second overlay with 0.004% Neutral Red applied after 48 hr incubation to enhance plaque visualization) in 6-well polystyrene culture plates. Sera that failed to neutralize the challenge dose of approximately 100 plaque-forming units of BCRV strain 80V8893 by 80% or more were considered seronegative. All sparrows were held for an initial 14-day quarantine period for acclimation while sera were being tested. Of the 30 birds, 26 were seronegative, and these were used for experimental inoculations or as controls. Twenty-four of the 26 seronegative sparrows were assigned randomly to control (hereafter CONTROL) or treatment groups (INOC) with an even representation of each sex among the groups. The four sparrows with evidence of previous exposure to BCRV were housed together in their own cage. The other 24 experimental birds were kept in six separate cages, with two INOC and two CONTROL birds in each one. All 28 birds were banded with uniquely marked plastic leg bands and were provided with ad libitum mixed bird seed and water. The experiment was conducted

TABLE 1. Summary of temporal patterns of viremia, viral RNA detection, and seroconversion data for Buggy Creek virus (BCRV) in house sparrows. Groups are birds that were experimentally inoculated with BCRV (INOC-5 and INOC-16), served as controls (CONTROL-5 and CONTROL-16), or were challenged with BCRV after testing positive for BCRV-reactive antibodies before the study (CHALLENGE).

	Temporal patterns of viremia		Viral RNA present[a]	Detection in tissue	Seroconversion
Group (sample size)	Maximum duration of viremia (days)	Maximum titer/titer range (log_{10}PFU/mL)	Duration range (days)/days postinoculation	Positive tissue types	No. seroconverting/titer range[b] [*n*]
INOC-5 (6)	3	5.0/2.7–5.0	1–3/d1–3	Brain, skin	5/40–160 [4]
CONTROL-5 (6)	None detected	NA[c]	None positive	None positive	0
INOC-16 (6)	Not tested	NA	6–10/d1, 7, 9–11, 14–15	Brain, skin	6/10–160 [5]
CONTROL-16 (6)	Not tested	NA	1–5/d9–15	None positive	0
CHALLENGE (4)	None detected	NA	None positive	Skin	2/20 [1]

[a] Based on RT-PCR detection in blood.

[b] "Titer range" refers to the range of 80% endpoint neutralization titers; sample sizes include only those birds with >80% neutralization.

[c] NA = not applicable.

under a winter light regime (14 hr dark:10 hr light).

The 12 birds assigned to the treatment group were inoculated subcutaneously on the breast with 100 μL of a 30,000 PFU/100 μL solution of BCRV. The inoculate strain, 80V8893 (ID no. B73266WSV), had been isolated from 50 male swallow bugs collected 30 August 1980 in Caddo County, Oklahoma, and passaged at least three times in Vero cell culture (Hopla et al., 1993). The 12 CONTROL sparrows were injected with 100 μL of a buffered saline solution. The four house sparrows that were initially seropositive were each "challenged" (hereafter CHALLENGE birds) with similar 100 μL subcutaneous doses of BCRV, to assess susceptibility in birds with BCRV-reactive antibodies.

Blood sampling and collection of oral swabs

We divided control and treatment groups according to two blood sampling schemes (see Table 1). Six birds of each group (INOC-5, CONTROL-5) and the four CHALLENGE birds were subjected to serial blood sampling of 50 μL each day for the first 5 days postinoculation using jugular venipuncture. These blood samples were stored in cryovials with 225 μL of BA-1 growth medium (containing M-199 Hank's salts, 1% bovine serum albumin, 0.05 M Tris-HCl [pH 7.5], 0.35 g/L sodium bicarbonate, and sterile water). Given our interest in assessing viremias over an extended period of time (>5 days) and limitations on the amount of blood that could be collected over that period, we sampled a droplet of blood (~10 μL) via brachial venipuncture from the remaining six birds in each group (INOC-16, CONTROL-16) over 16 days for a total of 15 samples (no sampling occurred on day 12). A droplet of blood was also retained from the 50 μL–serial blood samples for later comparison. All blood droplets were placed on 25 mm × 6 mm filter paper tabs (Whatman, Inc., Florham Park New Jersey, USA) and allowed to dry at room temperature until stored in closed Eppendorf tubes at −70 C for subsequent viral RNA extraction and analysis by reverse transcriptase-polymerase chain reaction (RT-PCR).

For all 24 experimental and control birds, nasopharyngeal (oral) swabs were collected through day 10 of the experiment. Dacron-tipped applicators were used, and swabs were placed in cryovials containing 500 μL BA-1 to transfer shed virus to the solution. All diluted blood samples and swabs were placed on wet ice at the time of sampling and later stored at −70 C.

Each day before sampling, all birds were examined for signs of illness or injury including recumbency, lethargy, emaciation, and deplumation. We recorded any fatalities and preserved carcasses at −70 C for later analysis. On day 16, 0.6 mL of blood was sampled from all living individuals (n=27). These samples were centrifuged to separate sera and stored at 4 C for testing for seroconversion. After final samples were obtained, all house sparrows were euthanized by carbon dioxide inhalation, and carcasses were stored at −70 C. All bird

capture, handling, blood sampling, swab collection, and euthanasia procedures were in accord with protocols approved by the Institutional Animal Care and Use Committee of the CDC's Division of Vector-Borne Infectious Diseases.

Tissue sampling

Following euthanasia, pieces of tissue up to 20 mm^3 were sampled from the brain, spleen, liver, lungs, and skin of all birds (except for the one that died on day 5 postinoculation). Skin samples were taken from the lower breast near the incision made to harvest internal organs. Instruments were cleaned with ethanol between removal of each organ from an individual. All tissue samples were placed in 1.0 mL of BA-1 with 20% fetal bovine serum supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 1 μg/mL Fungizone (Gibco-BRL, Gaithersburg, Maryland, USA) and macerated. The homogenates were placed in cryovials, centrifuged to clarify the solution, and stored at −70 C for subsequent analysis. RNA was extracted from 100 μL aliquots of the homogenate for each sample (see below).

Plaque assays

Viremia titers were determined by plaque assay for the 50 μL samples taken on days 1–5 for the six INOC-5 birds and four CHALLENGE sparrows. We added 100 μL of the serum solution in duplicate to monolayers of Vero cells in six-well polystyrene culture plates. We examined duplicates of six concentrations serially diluted in additional BA-1: undiluted, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}. Samples were incubated for 1 hr at 37.8 C in 5% CO_2 and then overlaid with 3 mL of 0.5% agarose in M-199 virus growth medium. The plates were returned to the incubator for 48 hr after which a second overlay including 0.004% Neutral Red dye was added for plaque visualization. Plaques were scored on day 3 after Vero cell infection. We used average plaque counts of the two replicate tests of each sample in calculating viremias reported here.

We assessed viral shedding by submitting 100 μL aliquots of the BA-1 swab storage solution to plaque assay using the protocol outlined above. Plaque assays were conducted on swab storage solution for swabs collected days 1 to 10 for all 12 INOC-5 and INOC-16 birds. To evaluate whether infectious BCRV persists in organs, we performed plaque assays (as described above) on 100 μL aliquots of the tissue homogenates that were positive by RT-PCR (see below).

RNA extraction and RT-PCR

Tissue samples were centrifuged at 11,000 × G for 1 min to clarify the supernatant and homogenates subsequently stored at −70 C. A 100 μL aliquot of the supernatant was added to 400 μL of a guanidine thiocyanate-based lysis buffer. Prior to RNA extraction, tissue samples were thawed and incubated at room temperature for 10 min. Paper tabs were also thawed to room temperature, and 400 μL of lysis buffer was added. Each paper-tab sample was vortexed for 30 sec. After the addition of 400 μL of 100% ethanol, RNA was extracted using the QIAmp Viral RNA Mini Kit (Qiagen Inc., Valencia, California, USA) following the manufacturer's protocol, modified by increasing the amount of buffer AVE (water) to yield a 100 μL RNA solution per sample. A negative control (water in place of supernatant but otherwise treated the same) was placed after every fifth sample during extraction and maintained in the same position for RT-PCR. A positive BCRV control was also included in each set of extractions.

RT-PCR was performed using the OneStep RT-PCR Kit (Qiagen Inc.) following the manufacturer's protocol. We used BCRV-specific primer sequences forward 5′-TAAGTTTGTTGGTCGAGAGCAGTATC-3′ and reverse 5′-ACACTCATAGGTAA-CAGTTTTTCCAGAC-3′, which yielded a 208-bp fragment from the E2 part of the viral genome (Moore et al., 2007). The cDNA amplification proceeded using the following conditions: reverse transcription for 30 min at 50 C, HotStarTaq (Qiagen) DNA polymerase activation for 15 min at 95 C, followed by 40 cycles of denaturation for 30 sec at 94 C, annealing for 30 sec at 60 C, elongation for 1 min at 72 C, and a final elongation (not cycled) for 10 min at 72 C. Product (6.5 μL) was electrophoresed on a 4% Nusieve© agarose gel to identify positive samples, using at least one BCRV positive control on each gel and a 100-bp ladder.

For all samples identified as positive by RT-PCR (i.e., bands present at 208 bp on agarose gels), RNA was reextracted and rerun in another RT-PCR with a second set of primers (forward 5′-TGGGCGAGATCTTTTAGC-GAGGAC-3′, reverse 5′-CAGAGACCCG-GAGGAATGATGC-3′) under the same thermocycling conditions. Paper tabs that were positive initially were also rerun in another RT-PCR using another aliquot of the initial RNA extraction and using the second set of primers. Only samples in which viral RNA was detected for both RT-PCR runs were considered positive for the analyses presented here.

Seroconversion

Final blood samples were tested for neutralizing antibodies as evidence of seroconversion by PRNT at a dilution of 1:10 and using approximately 100 PFU of BCRV strain 80V8893 in 6-well plates of Vero cells, overlaid with 0.5% agarose in M199 growth medium as outlined above. Samples with ≥80% reduction in Vero cell PFU were titrated in duplicate serial twofold dilutions to determine approximate endpoint titers of BCRV-reactive antibodies (through 1:320). Mean endpoint titers are reported here.

RESULTS

Of 12 experimentally inoculated house sparrows, 11 survived the duration of the 16-day study period; one bird (3581) died on day 5 postinoculation. All CONTROL house sparrows survived the duration of the experiment. One initially seropositive (CHALLENGE) bird died on day 9 postinoculation. No birds showed apparent signs of infection-related illness.

Temporal pattern of viremias

Among the INOC-5 birds, one bird (A1397) developed high levels of detectable viremia on days 1, 2, and 3 postinoculation. The maximum viremia detected (5.0 $\log_{10}$ PFU/mL) was on day 2 postinoculation for this bird. The maximum day 1 value was 3.8 $\log_{10}$ PFU/mL serum, and the day 3 maximum value was 4.3 $\log_{10}$ PFU/mL. Viral RNA was present in paper-tab samples from A1397 on days 1–3 as determined by RT-PCR. Serum from another bird (CDC0004) showed a lesser response to infection on day 1 postinoculation, where maximum viremia was 2.7 $\log_{10}$ PFU/mL. Paper-tab samples from a third bird in the INOC-5 group (3574) on days 1 and 2 were positive for the presence of viral RNA using RT-PCR. No viral RNA was detectable in paper-tab samples from any CONTROL-5 birds on any day (Table 1).

During the 16-day study period, viral RNA was detected in paper-tab samples from four of the six INOC-16 birds by RT-PCR on at least one day (Table 1). One (A1395) was positive on days 1, 7, 10, and 11. The other three (3484, 3575, and 3580) were positive on single days: day 9, day 14, and day 15, respectively.

Three of the six CONTROL-16 birds (A1399, A1398, and CDC0005) were positive by RT-PCR from paper tabs at some point during the study (Table 1). A1399 was positive on days 9, 10, and 14; A1398 was positive on days 9 and 13–15; and CDC0005 was positive on day 11.

Combining all paper-tab samples from INOC-16 and CONTROL-16 birds across all days, we found 1.7% of daily samples positive for BCRV on days 1–5 ($n=60$), versus 11.7% of daily samples from days 6–16 ($n=120$); this difference was statistically significant ($\chi^2_1=5.24$, $P=0.022$).

No plaque development was recorded from sera from the four CHALLENGE sparrows for any of the 5 study days (Table 1). Additionally, none of the paper-tab samples from these birds were positive by RT-PCR on days 1–5.

Virus shedding

Of the 12 experimentally inoculated birds, plaques developed from nasopharyngeal swab samples from two individuals. Individual A1397 (INOC-5) developed plaques on days 2, 6, and 10 with viral titers ranging from 1.8 $\log_{10}$ PFU/mL swab solution on day 2 to 1.0 $\log_{10}$ PFU/mL on day 10. The viral titer for individual 3577 (INOC-16) was 2.3 $\log_{10}$ PFU/mL on day 5.

Virus detection in tissue

BCRV RNA was detected in tissues of six of the 27 birds tested (Table 1). Brain and skin tissue of birds A1392 and A1393 (both INOC-5) were positive by RT-PCR; brain tissue of birds CDC0004 (INOC-5) and A1395 (INOC-16) was positive; and skin tissue of birds A1393 (INOC-16) and 3579 (CHALLENGE) was positive. No CONTROL birds showed evidence of BCRV in tissue by RT-PCR (Table 1). Buggy Creek virus was not detected in

spleen, lung, or liver tissue in any individuals. None of the aliquots of the tissue homogenate taken from the six samples identified as positive by RT-PCR developed detectable titers of virus by plaque assay.

Seroconversion

At the end of the 16-day experiment, all but one (3587) of the inoculated sparrows had seroconverted. Bird A1393 (INOC-16) had 50% neutralization at a titer of 10, while all other INOC-5 and INOC-16 individuals had >80% neutralization (n=9; Table 1). None of the CONTROL birds seroconverted. Two of the four CHALLENGE birds showed evidence of seroconversion; endpoint titers for the three surviving CHALLENGE birds were <10, 20, and 80, while the one that died on day 9 (3578) showed 50–60% plaque neutralization at a titer of 20.

DISCUSSION

We found evidence of BCRV viremia or presence of viral RNA in seven of 12 inoculated house sparrows, demonstrating the potential for this virus to replicate in these vertebrate hosts. Some infected sparrows shed BCRV orally. Detection of BCRV RNA in brain and skin tissue at 15 days after inoculation suggests enduring viral replication in those tissues, although we failed to isolate infectious viral particles from tissue other than blood. The discovery of BCRV RNA in blood of noninoculated (CONTROL) individuals suggests possible bird-to-bird (contact) transmission. However, only one of six inoculated individuals for which plaque assay was performed showed evidence of viremia sufficiently high to potentially infect hematophagous invertebrate vectors, based on similar studies in mosquitoes.

BCRV transmission and maintenance

Our results for BCRV in house sparrows suggest a viremia profile largely consistent with that reported for other alphaviruses, in which virus is most easily detected in blood for typically 1–2 days after initial infection (Hardy and Reeves, 1990; Komar et al., 1999; Lindström and Lundström, 2000; Kramer et al., 2002; Reisen et al., 2003). That all but one inoculated sparrow seroconverted suggests that our experimental infections were successful, but, perhaps surprisingly, only one of six sparrows showed high levels of viremia, and four of the six inoculated sparrows showed no evidence of viremia by plaque assay. Interestingly, we found virus RNA detectable in blood by RT-PCR for a much longer period of time, up to 15 days postinoculation, than expected based on similar studies with alpha- and flaviviruses. This may reflect in general the greater sensitivity of RT-PCR to detect the presence of viral RNA (Kramer et al., 2002). Birds were more likely to be positive for BCRV by RT-PCR on days 6–15 than on days 1–5, a perplexing result given that virus titers in most bird species are highest immediately after infection (e.g., Hardy and Reeves, 1990; Komar et al., 1999; Kramer et al., 2002; Reisen et al., 2003). The RT-PCR detections through day 15 may simply indicate low levels of virus (or viral RNA) in blood that are not sufficiently strong to be detectable by plaque assay or, more importantly, sufficiently strong or viable to infect invertebrate vectors.

The plaque assay results from days 1–3 still suggest, however, that house sparrows have the capacity to serve as an amplifying host for BCRV. That four of 30 birds caught at a feedlot in winter (located about 2.8 km from a cliff swallow colony of an estimated 1,100 nests) had serological evidence of possible exposure to BCRV also suggests that sparrows are often exposed and may help maintain the virus in an enzootic state. Determining the house sparrow's relative role and competence as an amplifying host compared to cliff swallows must await comparative data for the latter species. However, because

nonmigratory house sparrows are permanent residents in North America and potentially present at cliff swallow colony sites for a longer period of time each year than cliff swallows, they have a longer exposure to swallow bugs and thus potentially to the BCRV vectored by the bugs. In addition, sparrows are present at many colony sites in summers when cliff swallows are absent at a given site (perhaps because bug infestations are too high; Brown and Brown, 1996), and the ability of sparrows to amplify the virus may help maintain it between cliff swallow occupancy bouts at a given locality. Although we know that swallow bugs feed on house sparrows, we do not know the relative extent to which the bugs take blood meals from sparrows (compared to cliff swallows) or the degree to which sparrow behavior (e.g., the filling of swallow nests with grass) affects bug feeding behavior. This sort of information is needed to fully evaluate how important house sparrows may be in the transmission and maintenance of BCRV.

The highest level of viremia we detected in a house sparrow was 5.0 $\log_{10}$ PFU/mL of serum on day 2 postinoculation, but the threshold dose for per os infection of swallow bugs remains unknown. Viremias above 6.5 $\log_{10}$ PFU/mL were required to infect mosquitoes with the alphaviruses Getah virus (Turell et al., 2006) and Venezuelan equine encephalitis virus (Ortiz et al., 2005). However, for WEEV (more closely related to BCRV), titers of 2.6–3.5 $\log_{10}$ PFU/mL were sufficient to infect some mosquitoes (Mahmood et al., 2006), suggesting that the lower viremia titers found for BCRV in this study might be sufficient to infect invertebrate vectors under some circumstances. On the other hand, the degree of WEEV transmission by experimentally infected mosquitoes was low: 4–21% of those infected subsequently transmitted (Mahmood et al., 2006), suggesting that efficient transmission of WEEV requires higher doses of the virus.

Bird-to-bird transmission

Our experiment provided some evidence of bird-to-bird transmission of BCRV. Two of the CONTROL birds (A1398, CDC0005) with evidence of BCRV RNA in blood (by RT-PCR) were each housed with one of the two inoculated individuals that showed BCRV viremia by plaque assay (birds A1397 and CDC0004, respectively), and the other positive CONTROL bird (A1399) was housed with an inoculated individual (3575) that was positive by RT-PCR. That we found evidence of plaque-forming virus in nasopharyngeal swabs from inoculated individuals suggests that infectious BCRV can be transmitted orally among sparrows, and perhaps the control birds were exposed through use of watering or food trays shared with inoculated birds or through other contact with virus shed orally (Holden, 1955; Komar et al., 2003). The presence of BCRV in skin also provides another potential mechanism of bird-to-bird transmission, if individuals peck each other during aggressive interactions and potentially break the skin. Although use of common food and water sources by confined individuals may be an artifact of captivity, the presumed ability of BCRV to be passed from bird to bird means that the dense concentrations of cliff swallows at nesting colonies may provide unusually good opportunities for non–vector-borne transmission of this alphavirus. Strong evidence for bird-to-bird transmission of EEEV, another alphavirus, was detected among pheasants that shared the same pens with inoculated birds (Holden, 1955).

The CONTROL birds that tested positive for BCRV showed evidence of viral RNA from paper tabs. Only those that confirmed twice by RT-PCR (using different primers each time) were scored as positive, but we should note that both RT-PCR reactions for an individual used aliquots of RNA from the same extraction. Because we had only one paper tab from each individual, we could not do a second,

independent RNA extraction for each putatively positive bird. Thus, we could not completely rule out laboratory contamination during the extraction procedure, and this limitation also applied to the paper-tab samples from the INOC-16 birds. Contamination might be suggested by the fact that none of the three CONTROL birds that tested positive for BCRV by RT-PCR had seroconverted by the end of the experiment. However, because none of these birds showed evidence of exposure by RT-PCR before day 9, not enough time would have elapsed for them to seroconvert before the end of the 16-day experiment. Furthermore, there was no tangible evidence of any contamination (all negative controls were clean), and we doubt that the results can be explained by laboratory contamination. Reeves et al. (1958) also found evidence that bird-to-bird transmission of an alphavirus (WEEV) might have occurred among individuals housed in an aviary, but they too could not completely rule out methodological error.

Detection of BCRV in tissue

We found evidence of BCRV in the brain and skin tissue of about 25% of those birds inoculated. The related WEEV has been found to persist in brain, blood, lung, liver, spleen, and gall bladder tissue of birds for up to 10 months after infection (Reeves et al., 1958; Reisen et al., 2001). Maintenance of chronic virus infections frequently occurs in vertebrates (Kuno, 2001). Relapse of latent virus infection may account for arbovirus overwintering and local persistence of infection among vertebrates within a transmission focus, a scenario well suited for the sedentary house sparrows that live in or near cliff swallow colonies. Evidence to date, however, has shown that most viruses latent in avian tissue rarely activate to a highly infectious state (Reisen et al., 2001, 2003). Our study was not designed to study persistence of BCRV over an extended time period, but we did find viral RNA still present in avian tissue (including blood) 16 days after inoculation (when the study ended). Persistent alphavirus infection in skin has not been previously reported, to our knowledge. If infectious BCRV occurs in skin, it could contaminate the mouthparts of blood-feeding swallow bugs, providing an alternative mechanism for infecting the vectors.

Effects of BCRV on house sparrows

How BCRV affects its vertebrate hosts (cliff swallows, house sparrows) is unknown, because no studies have addressed this question. In our experiment two of 16 inoculated house sparrows (INOC and CHALLENGE) died within 10 days of inoculation, whereas none of the 12 control birds succumbed. This difference was not significant (χ^2_1, $P=0.20$); however, it is suggestive that BCRV can negatively affect birds at times. On the other hand, we saw no evidence of behavioral differences that might reflect sickness between inoculated and control individuals. The degree of mortality, 12.5%, we observed among inoculated birds for BCRV is less than the overall 33% mortality seen among eight bird species for the related WEEV (Hardy and Reeves, 1990), although mortality can vary widely depending on the virus strain used in the inoculations and host species (Reisen et al., 2003). Possibly BCRV is more like St. Louis encephalitis virus (*Flaviviridae*), in which avian mortality due to the virus is apparently quite low (McLean and Bowen, 1980), contributing to the virus's annual enzootic persistence.

In summary, this study shows that introduced house sparrows can serve as a host for BCRV and additional studies may demonstrate that sparrows are important amplifying hosts for BCRV. The ability of BCRV to replicate in sparrows and the low apparent mortality associated with infection may contribute to the persistence of this virus at sites in the absence of cliff swallows. Our study also suggested that

BCRV can be passed from bird to bird, and if true, this virus may be well adapted to the high density of cliff swallows that occur in many of the breeding colonies.

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LITERATURE CITED

Beaty, B. J., C. R. Calisher, and R. E. Shope. 1995. Arboviruses. *In* Diagnostic procedures for viral, rickettsial, and chlamydial infections, 7th Edition, E. H. Lenette, D. A. Lenette, and E. T. Lenette (eds.). American Public Health Association, Washington, D.C., pp. 189–212.

Brown, C. R., and M. B. Brown. 1996. Coloniality in the cliff swallow: The effect of group size on social behavior. University of Chicago Press, Chicago, Illinois.

———, N. Komar, S. B. Quick, R. A. Sethi, N. A. Panella, M. B. Brown, and M. Pfeffer. 2001. Arbovirus infection increases with group size. Proceedings of the Royal Society of London B 268: 1833–1840.

———, M. B. Brown, A. T. Moore, and N. Komar. 2007. Bird movement predicts Buggy Creek virus infection in insect vectors. Vector-Borne and Zoonotic Diseases 7: 304–314.

Calisher, C. H., K. S. C. Maness, R. D. Lord, and P. H. Coleman. 1971. Identification of two South American strains of eastern equine encephalomyelitis virus from migrant birds captured on the Mississippi delta. American Journal of Epidemiology 94: 172–178.

Hardy, J. L., and W. C. Reeves. 1990. Experimental studies on infection in vertebrate hosts. *In* Epidemiology and control of mosquito-borne arboviruses in California, 1943–1987, W. C. Reeves (ed.). California Mosquito and Vector Control Association, Sacramento, California, pp. 66–127.

Holden, P. 1955. Transmission of eastern equine encephalomyelitis in ring-necked pheasants. Proceedings of the Society for Experimental Biology and Medicine 88: 607–610.

Hopla, C. E., D. B. Francy, C. H. Calisher, and J. S. Lazuick. 1993. Relationship of cliff swallows, ectoparasites, and an alphavirus in west-central Oklahoma. Journal of Medical Entomology 30: 267–272.

Komar, N., D. J. Dohm, M. J. Turell, and A. Spielman. 1999. Eastern equine encephalitis virus in birds: Relative competence of European starlings (*Sturnus vulgaris*). American Journal of Tropical Medicine and Hygiene 60: 387–391.

———, S. Langevin, S. Hinten, N. Nemeth, E. Edwards, D. Hettler, B. Davis, R. Bowen, and M. Bunning. 2003. Experimental infection of North American birds with the New York 1999 strain of West Nile virus. Emerging Infectious Diseases 9: 311–322.

Kramer, L. D., T. M. Wolfe, E. N. Green, R. E. Chiles, H. Fallah, Y. Fang, and W. K. Reisen. 2002. Detection of encephalitis viruses in mosquitoes (Diptera: Culicidae) and avian tissues. Journal of Medical Entomology 39: 312–323.

Kuno, G. 2001. Persistence of arboviruses and antiviral antibodies in vertebrate hosts: Its occurrence and impacts. Reviews in Medical Virology 11: 165–190.

Lindström, K., and J. Lundström. 2000. Male greenfinches (*Carduelis chloris*) with brighter ornaments have higher virus clearance rate. Behavioral Ecology and Sociobiology 48: 44–51.

Lord, R. D., and C. H. Calisher. 1970. Further evidence of southward transport of arboviruses by migratory birds. American Journal of Epidemiology 92: 73–78.

Lowther, P. E., and C. L. Cink. 1992. House sparrow. *In* The birds of North America, A. Poole, P. Stettenheim, and F. Gill (eds.). Academy of Natural Sciences, Philadelphia, Pennsylvania, and American Ornithologists' Union, Washington, D.C., no. 12.

Mahmood, F., R. E. Chiles, Y. Fang, E. N. Green, and W. K. Reisen. 2006. Effects of time after infection, mosquito genotype, and infectious viral dose on the dynamics of *Culex tarsalis* vector competence for western equine encephalomyelitis virus. Journal of the American Mosquito Control Association 22: 272–281.

McLean, R. G., and G. S. Bowen. 1980. Vertebrate hosts. *In* St. Louis encephalitis, T. P. Monath (ed.). American Public Health Association, Washington, D.C., pp. 381–450.

Moore, A. T., E. A. Edwards, M. B. Brown, N. Komar, and C. R. Brown. 2007. Ecological correlates of Buggy Creek virus infection in cimicid swallow bugs *Oeciacus vicarius*, southwestern Nebraska, 2004. Journal of Medical Entomology 44: 42–49.

Ortiz, D. I., M. Anischenko, and S. C. Weaver.

2005. Susceptibility of *Psorophora confinnis* (Diptera: Culicidae) to infection with epizootic (subtype IC) and enzootic (subtype ID) Venezuelan equine encephalitis viruses. Journal of Medical Entomology 42: 857–863.

PFEFFER, M., J. E. FOSTER, E. A. EDWARDS, M. B. BROWN, N. KOMAR, AND C. R. BROWN. 2006. Phylogenetic analysis of Buggy Creek virus: Evidence for multiple clades in the western Great Plains, United States of America. Applied and Environmental Microbiology 72: 6886–6893.

RAPPOLE, J. H., AND Z. HUBÁLEK. 2003. Migratory birds and West Nile virus. Journal of Applied Microbiology 94: 47S–58S.

REEVES, W. C. 1974. Overwintering of arboviruses. Progress in Medical Virology 17: 193–220.

———, G. A. HUTSON, R. E. BELLAMY, AND R. P. SCRIVANI. 1958. Chronic latent infections of birds with western equine encephalomyelitis virus. Proceedings of the Society for Experimental Biology and Medicine 97: 733–736.

REISEN, W. K., AND T. P. MONATH. 1989. Western equine encephalomyelitis. *In* The arboviruses: Epidemiology and ecology, Vol. 5, T. P. Monath (ed.). CRC Press, Boca Raton, Florida, pp. 89–137.

———, L. D. KRAMER, R. E. CHILES, E.-G. N. GREEN, AND V. M. MARTINEZ. 2001. Encephalitis virus persistence in California birds: Preliminary studies with house finches. Journal of Medical Entomology 38: 393–399.

———, R. E. CHILES, V. M. MARTINEZ, Y. FANG, AND E. N. GREEN. 2003. Experimental infection of California birds with western equine encephalomyelitis and St. Louis encephalitis viruses. Journal of Medical Entomology 40: 968–982.

RUSH, W. A., D. B. FRANCY, G. C. SMITH, AND C. B. CROPP. 1980. Transmission of an arbovirus by a member of the family Cimicidae. Annals of the Entomological Society of America 73: 315–318.

SCOTT, T. W., G. S. BOWEN, AND T. P. MONATH. 1984. A field study on the effects of Fort Morgan virus, an arbovirus transmitted by swallow bugs, on the reproductive success of cliff swallows and symbiotic house sparrows in Morgan County, Colorado, 1976. American Journal of Tropical Medicine and Hygiene 33: 981–991.

STAMM, D. D., AND R. J. NEWMAN. 1963. Evidence of southward transport of arboviruses from the U.S. by migratory birds. Annals of Microbiology 11: 123–133.

STRAUSS, J. H., AND E. G. STRAUSS. 1994. The alphaviruses: Gene expression, replication, and evolution. Microbiology Reviews 58: 491–562.

TURELL, M. J., C. N. MORES, D. J. DOHM, W.-J. LEE, H.-C. KIM, AND T. A. KLEIN. 2006. Laboratory transmission of Japanese encephalitis, West Nile, and Getah viruses by mosquitoes (Diptera: Culicidae) collected near Camp Greaves, Gyeonggi Province, Republic of Korea, 2003. Journal of Medical Entomology 43: 1076–1081.

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