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Amy T. Moore
University of Tulsa

Eric A. Edwards
Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention

Mary Bomberger Brown
University of Tulsa, mbrown9@unl.edu

Nicholas Komar
Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, nck6@cdc.gov

Charles R. Brown
University of Tulsa, charles-brown@utulsa.edu

Ecological Correlates of Buggy Creek Virus Infection in *Oeciacus vicarius*, Southwestern Nebraska, 2004

AMY T. MOORE,[1] ERIC A. EDWARDS,[2] MARY BOMBERGER BROWN,[1] NICHOLAS KOMAR,[2] AND CHARLES R. BROWN[1,3]

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ABSTRACT Buggy Creek virus (family *Togaviridae*, genus *Alphavirus*, BCRV) is an alphavirus within the western equine encephalitis virus complex whose primary vector is the swallow bug, *Oeciacus vicarius* Horvath (Hemiptera: Cimicidae), an ectoparasite of the colonially nesting cliff swallow, *Petrochelidon pyrrhonota*, that is also a frequent host for the virus. We investigated ecological correlates of BCRV infection in 100-bug pools at 14 different swallow colony sites in southwestern Nebraska from summer 2004, by using plaque assay on Vero cells to identify cytopathic virus and reverse transcription-polymerase chain reaction to identify noncytopathic viral RNA. We found 26.7% of swallow bug pools positive for BCRV, with 15.6% showing cytopathic ("infectious") virus and 11.0% noncytopathic ("noninfectious") viral RNA. The prevalence of cytopathic BCRV increased with cliff swallow colony size in the current year; the percentage of noncytopathic samples at a site did not vary with colony size in the current year but increased with the previous year's colony size at a site. Active colony sites (those used by swallows) had higher percentages of cytopathic BCRV in bug pools than at inactive colony sites, but the reverse held for noncytopathic viral RNA. Nests that were occupied by birds at some time in the season had more pools with cytopathic BCRV than did inactive nests. Colonies used by birds for the first or second time had less virus in bugs than did sites that had had a longer history of bird use. The percentage of pools with BCRV was affected by whether bugs were clustering at nest entrances or distributed elsewhere on a nest. The prevalence of cytopathic samples decreased at inactive colony sites and increased at active sites over the course of the summer, whereas the reverse pattern held for noncytopathic samples. Noncytopathic bug pools seem to reflect infection patterns from a previous year. The results suggest that the birds play an important role in amplification of the virus and that the spatial foci of BCRV occurrence can be predicted based on characteristics of cliff swallow colonies and the cimicid bugs that are associated with them.

KEY WORDS cliff swallow, coloniality, noninfectious virus, parasitism, virus ecology

Buggy Creek virus (family *Togaviridae*, genus *Alphavirus*, BCRV) is a recombinant alphavirus within the western equine encephalitis virus antigenic complex (Hayes et al. 1977; Calisher et al. 1980, 1988; Monath et al. 1980). The principal known invertebrate vector for BCRV is the blood-feeding swallow bug, *Oeciacus vicarius* Horvath (Hemiptera: Cimicidae), an ectoparasite of the colonially nesting cliff swallow, *Petrochelidon pyrrhonota* (Rush et al. 1980, 1981; Hopla et al. 1993, Brown et al. 2001). This virus is unusual in being one of the few alphaviruses routinely vectored by an invertebrate other than mosquitoes (Strauss and Strauss 1994). Because the wingless swallow bugs are largely sedentary and confined during much of the year to occupied and unoccupied cliff swallow nests (Loye 1985; Brown and Brown 2004, 2005), the spatial foci for BCRV presence are predictable. This allows one to relate site characteristics such as swallow colony size or bug population size to the likelihood of virus persistence at a site (Brown et al. 2001).

Previous work has primarily focused on how BCRV infection in *O. vicarius* varies as a function of the number of birds and bugs at active colony sites (Brown et al. 2001). These studies showed that the percentages of bug pools positive for cytopathic virus increased with swallow colony size; and for colonies active in successive years, the percentages of pools positive tended to autocorrelate between consecutive seasons (Brown et al. 2001). However, we know relatively little about other ecological relationships between BCRV and cliff swallow or bug population biology. For example, does a colony's or a specific nest's occupancy status by birds affect BCRV in bugs? Bugs are present in unoccupied cliff swallow nests and at unused colony sites (Brown and Brown 1996), yet to date we have little information on how the presence or absence of birds influences the occurrence of BCRV. When cliff swallows occupy a colony site for the first time, is

[1] Department of Biological Sciences, University of Tulsa, Tulsa, OK 74104.
[2] Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, P.O. Box 2087, Fort Collins, CO 80522.
[3] Corresponding author, e-mail: charles-brown@utulsa.edu.

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BCRV introduced with the first bugs that colonize the site?

The objective of this study is to explore patterns of BCRV infection among swallow bug vectors at 14 different cliff swallow colonies of different sizes and occupancy status during the 2004 summer season at our southwestern Nebraska study site. Previous work (Brown et al. 2001) used exclusively plaque assay to detect BCRV and thus studied only cytopathic virus. In this study we used plaque assay and reverse transcription-polymerase chain reaction (RT-PCR) to screen bug samples, and the latter methodology allowed us to also detect noncytopathic viral RNA in bug samples. Our objectives were to better understand in general the ecology of BCRV in this population of hosts and vectors and to explore ecological differences associated with the presence of cytopathic versus noncytopathic viral RNA in bugs.

Materials and Methods

Study Organisms. Buggy Creek virus was first isolated in 1980 from *O. vicarius* collected at a cliff swallow colony along Buggy Creek in Grady County, west central Oklahoma (Loye and Hopla 1983, Hopla et al. 1993). BCRV is very similar to another alphavirus, Fort Morgan virus (family *Togaviridae*, genus *Alphavirus*, FMV), which also is associated with cliff swallows and *O. vicarius* (Hayes et al. 1977, Calisher et al. 1980, Scott et al. 1984). BCRV and FMV are strains of the same virus, based on nucleotide similarities and phylogenetic analyses (Pfeffer et al., 2006).

The hematophagous *O. vicarius* is an ectoparasite primarily of cliff swallows. It is found throughout the bird's wide geographic range (Brown and Brown 1995). Swallow bugs are nest-based parasites that overwinter in cliff swallows' nests or in the cracks and crevices of the nesting substrate near the nests. Infestations can reach 2,600 bugs per nest, and the bugs affect many aspects of cliff swallow life history (Brown and Brown 1986, 1992, 1996, Chapman and George 1991, Loye and Carroll 1991). Swallow bugs begin to reproduce as soon as they feed in the spring. Eggs are laid in several clutches that hatch over variable lengths of time, ranging from 3 to 5 d (Loye 1985) to 12 to 20 d (Myers 1928). Bug populations at an active colony site increase throughout the summer, reaching a peak at approximately the time cliff swallows fledge. The bugs seem to be adapted to withstanding long periods of host absence, in some cases persisting at a site not used by cliff swallows for up to at least three consecutive years (Smith and Eads 1978, Loye 1985, Loye and Carroll 1991, Rannala 1995). Bugs seem capable of parasitizing introduced house sparrows, *Passer domesticus*, that occupy nests in some cliff swallow colonies (Hopla et al. 1993, Brown et al. 2001; C.R.B. et al., personal observation).

Cliff swallows are highly colonial passerines that breed commonly in western North America and more rarely farther east (Brown and Brown 1995). They build gourd-shaped mud nests and attach them to the vertical faces of cliff walls, rock outcrops, or artificial sites such as the eaves of buildings or bridges. Their nests tend to be stacked closely together, often sharing walls. Cliff swallows are migratory, wintering in southern South America, and they have a relatively short breeding season in North America. They begin to arrive at our study site in late April or early May and depart by late July. Most birds raise only one brood.

Study Site. Our study site is centered at the Cedar Point Biological Station (41° 13′ N, 101° 39′ W) near Ogallala, in Keith County, along the North and South Platte rivers, and also includes portions of Deuel, Garden, and Lincoln counties, southwestern Nebraska. Cliff swallows have been studied there since 1982. Approximately 160 cliff swallow colony sites are in our 150- by 50-km study area, and about one-third are not used in a given year. Colony size varies widely; in our study area, it ranges from 2 to 6,000 nests, with some birds nesting solitarily. Over a 20-yr period, mean ± SE colony size (n = 1,363) was 363 ± 16 nests. Each colony site tends to be separated from the next nearest by 1 to 10 km but in a few cases by ≥20 km. The study site is described in detail by Brown and Brown (1996).

Cliff swallow colony size was defined as the maximum number of nests at a site to have contained one or more eggs. Active nests were counted at some sites by periodically checking the nest contents with a dental mirror and flashlight, whereas the colony size at other sites was estimated by counting the total number of nests in sections of the colony known to be active. Full details on these methods of determining colony sizes are given in Brown and Brown (1996).

Field Collections of Bugs. In summer 2004, all *O. vicarius* were collected from the outsides of cliff swallow nests. Bugs generally either clustered just inside the tubular entrances of the nests in a relatively dense mass, or they were distributed in typically lower density across the bottom and sides of the nests and below the entrance. Bugs that cluster at the entrances are mostly adults, and this behavior seems to be to facilitate dispersal from a nest (when a transient bird passes by and makes physical contact with the nest); consequently, clustering usually occurs at nests and colony sites unoccupied by birds. Bugs at active nests do not cluster at entrances and instead spread out over the outside surface where they lay eggs and rest in between bloodmeals. We brushed bugs off nests into a wide-mouthed collecting jar by using a wire brush, and we noted whether the collections were of clustering bugs or ones on the outside surface and whether the nest was active or inactive. We collected from throughout a colony site (in parts where nests were accessible), but only took from nests where bugs were visible to us (i.e., no nests were collected, and thus no bugs from inside or behind the nests were included). We attempted to collect a minimum of 1,000 bugs per site and sampled 10–30 nests depending on the level of infestation at a site. Most colony sites were sampled once (on one date) during the summer, but when an inactive site later became active, samples were taken on a second date. Bugs were transferred from the collecting jar to plastic bags, transported to the Cedar Point Biological Station, and sorted into pools of 100

individuals while alive. Pools were frozen immediately after sorting at −70°C.

Previous work (Brown et al. 2001) separated *O. vicarius* pools into adults and instars. These age classes can be identified partly by the instars' smaller size (Usinger 1966). However, because Brown et al. (2001) found no difference in BCRV prevalence between the age classes, and because of the difficulty in accurately identifying and separating the later stage instars from adults, we combined the two age classes in the analyses that follow.

Virus Screening and Isolation. Bug pools were macerated by mortar and pestle and suspended in 1.0 ml of BA-1, a growth medium containing antibiotics and 20% fetal bovine serum. The homogenate was 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. Before RNA extraction, samples were thawed and incubated at room temperature for 10 min. After the addition of 400 μl of 100% ethanol, RNA was extracted using the QIAmp viral RNA mini kit (QIAGEN, Valencia, CA) following the manufacturer's protocol, modified by increasing the amount of buffer AVE (water) to yield 100 μl total RNA per sample. A negative control (water in place of supernatant but otherwise treated the same) was placed between every five samples during extraction and maintained in the same position for RT-PCR. A positive BCRV control also was included in each extraction.

RT-PCR was performed using the One-Step RT-PCR kit (QIAGEN) following the manufacturer's protocol. We used BCRV-specific primer sequences forward 5′-TAAGTTTGTTGGTCGAGAGCAGTATC-3′ and reverse 5′-ACACTCATAGGTAACAGTTTTTC CAGAC-3′, which yielded a 208-bp fragment from the E2 part of the viral genome. Thermocycler conditions were RT for 30 min at 50°C, HotStarTaq DNA polymerase activation for 15 min at 95°C, followed by 40 cycles of denaturation for 30 s at 94°C, annealing for 30 s 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 pools, by using at least one BCRV-positive control on each gel and a 100-bp ladder.

All samples were screened initially with RT-PCR, and negatives were identified from the initial screen. All positive pools were subjected to plaque assay in Vero cells. We added 100 μl of the supernatant in duplicate to a monolayer of Vero cells in a six-well cell culture plate, incubated it for 1 h at 37.8°C in 5% CO_2, and then overlaid each monolayer with 3 ml of 0.5% agarose in M-199 medium supplemented with 350 mg/liter sodium bicarbonate, 29.2 mg/liter L-glutamine, and antibiotics and returned the plate to the incubator. A second overlay containing 0.004% neutral red dye was added after 2-d incubation for plaque visualization. Plaques were scored daily for 5 d, with the titer expressed as plaque-forming units per 0.1 ml. For samples showing no plaques on Vero cells, we reextracted RNA from the remaining homogenate and performed RT-PCR again (with the same primers) for confirmation. A pool was considered to contain cytopathic BCRV (termed "infectious" by some) only if it showed positive both by the initial RT-PCR and by plaque assay. A pool was considered to contain noncytopathic BCRV RNA (termed "noninfectious" by some) if it tested positive twice by RT-PCR but showed no plaque development on Vero cells.

Results

In 2004, 26.7% of swallow bug pools tested positive for BCRV (n = 390). Those with cytopathic virus accounted for 15.6% of the total pools, and those with noncytopathic viral RNA accounted for 11.0% of the total.

Effect of Colony Size. The percentage of bug pools positive for cytopathic BCRV increased with swallow colony size in summer 2004 (Fig. 1). The relationship was significant when using all colony sites, and when the single site with $n < 10$ pools (in this case, 6) was excluded, the correlation was even stronger (Fig. 1). There was no significant relationship between colony size in summer 2004 and the percentage of pools with noncytopathic viral RNA, regardless of whether the site with a small sample was included (Fig. 1). However, the percentage of pools positive for noncyto-

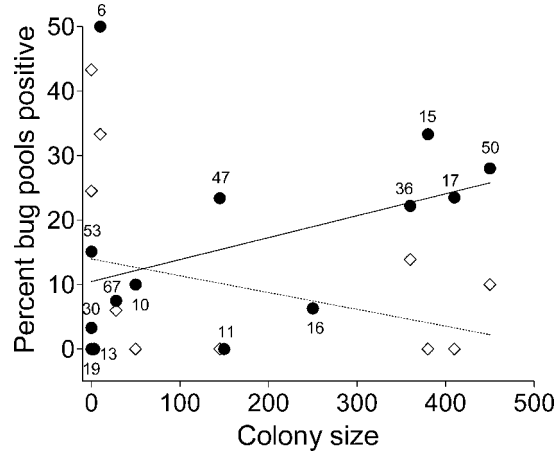

Fig. 1. Percentage of swallow bug pools positive for cytopathic BCRV (●) and noncytopathic viral RNA (◇) at a site in relation to cliff swallow colony size (number of nests) in the current year. The percentage of pools positive for cytopathic BCRV increased significantly with colony size for all sites (r_s = 0.52, P = 0.05, n = 14 colonies) and when a site with a sample size of <10 pools was excluded (r_s = 0.68, P = 0.010, n = 13 colonies). The percentage of pools positive for noncytopathic viral RNA did not vary with colony size for all sites (r_s = −0.24, P = 0.40, n = 14) or when the site with a small sample size was excluded (r_s = −0.16, P = 0.61, n = 13). Numbers by symbols show the total number of pools analyzed for each colony site. The lines represent best-fit linear least-squares regressions, for cytopathic (solid line) and noncytopathic samples (dotted line). Data (◇) for some sites are hidden by similar values overlaying them.

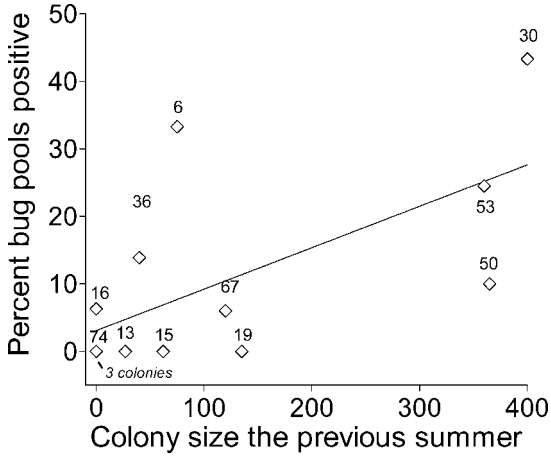

Fig. 2. Percentage of swallow bug pools positive for noncytopathic viral RNA at a site in relation to cliff swallow colony size (number of nests) in the previous year. The percentage of pools positive increased significantly with colony size for all sites ($r_s = 0.61$, $P = 0.028$, $n = 13$ colonies) and when a site with a sample size of <10 pools was excluded ($r_s = 0.62$, $P = 0.03$, $n = 12$ colonies). Numbers by symbols show the total number of pools analyzed for each colony site. The line represents a best-fit linear least-squares regression.

pathic viral RNA increased significantly with swallow colony size the previous summer (2003) (Fig. 2). For these analyses, a colony site unused by birds in a given year had a "0" colony size.

There was no significant correlation between the percentage of pools positive for cytopathic BCRV at a site and those positive for noncytopathic viral RNA at the same site during summer 2004 ($r_s = 0.22$, $P = 0.45$, $n = 14$ colony sites).

Effect of Colony and Nest Occupancy by Birds. At the time of sampling bugs, each colony site was considered either active (nesting birds present), later active (no birds present but the site had nesting birds later that summer), or inactive (site was abandoned all summer). Active ($n = 250$ pools), later active ($n = 39$), and inactive ($n = 100$) colonies had 20.4, 2.6, and 9.0 pools positive for cytopathic BCRV, respectively, and 6.4, 2.6, and 26.0% pools positive for noncytopathic viral RNA, respectively. The percentages differed significantly between the colony types ($\chi^2_4 = 41.7$, $P < 0.00001$). Because later active and inactive colony sites both had no birds present at the time of sampling, we tested whether these differed in the proportion of pools positive for cytopathic and noncytopathic BCRV and found a significant difference ($\chi^2_2 = 13.0$, $P = 0.001$).

Nests were assigned to one of three categories at the time bugs were sampled: active (containing eggs or nestlings), formerly active (birds had been present earlier but the nestlings had either fledged or died, and the parents had abandoned), or inactive (not currently used by birds and not used earlier that summer). Active ($n = 174$ pools), formerly active ($n = 54$), and inactive ($n = 161$) nests had 19.0, 33.3, and 6.2% pools positive for cytopathic BCRV, respectively, and 4.6, 9.3, and 18.6% pools positive for noncytopathic BCRV, respectively. The percentages differed significantly between the nest types, both overall ($\chi^2_4 = 38.4$, $P < 0.00001$) and when comparing formerly active to inactive nests ($\chi^2_2 = 26.8$, $P < 0.00001$).

Because colony status and nest status were confounded to some degree (inactive colony sites could have only inactive nests), we also analyzed the percentage of positive pools in relation to nest status by colony for three sites where we had at least two categories of nests. At one site, active ($n = 33$ pools) and formerly active ($n = 13$) nests had 18.2 and 38.5% pools positive for cytopathic BCRV, respectively (no noncytopathic viral RNA was detected); at another, formerly active ($n = 40$ pools) and inactive ($n = 10$) nests had 32.5 and 10.0% pools positive for cytopathic BCRV, respectively, and 12.5 and 0% pools positive for noncytopathic viral RNA, respectively; and at a third, active ($n = 35$ pools) and inactive ($n = 32$) nests had 14.3 and 0% pools positive for cytopathic BCRV, respectively, and 2.9 and 9.4% pools positive for noncytopathic viral RNA, respectively. The separate percentages for these three sites were generally consistent with the overall pattern across all colonies.

One colony site, of ≈150 active nests, was used by cliff swallows for the first time in 2004, being in a newly constructed highway culvert. All active nests there were built by the birds in 2004. Another newly constructed culvert was used by the birds for the first time in 2003 (≈135 active nests) but unused in summer 2004. Thus, these "new" sites had only been used by cliff swallows for one season. We found no pools positive for either cytopathic or noncytopathic virus ($n = 30$) from either of these colony sites in summer 2004, a significant difference from the distribution at all sites ($n = 344$ pools) that had been used in two or more past years ($\chi^2_2 = 12.2$, $P = 0.002$). One active colony site sampled in 2004 had been used by birds in only one previous year (2002, when it contained 335 nests), and at that site the percentage of bug pools positive for both cytopathic BCRV and noncytopathic viral RNA was 6.3% ($n = 16$ pools).

Effect of Bug Behavior. For bugs that were clustering at nest entrances ($n = 215$ pools), 13.0% of pools were positive for cytopathic BCRV and 16.3% were positive for noncytopathic viral RNA. For bugs collected from the outside surface of nests ($n = 174$ pools), 19.0% of pools were positive for cytopathic BCRV and 4.6% were positive for noncytopathic viral RNA. The percentage differences between the behavior types were significant ($\chi^2_2 = 14.5$, $P < 0.001$). Because inactive colonies contained only clustering bugs, behavior and colony occupancy status were confounded. Only at active colonies did bugs exhibit both behavior types within the same site (albeit at different nests). For active colonies only, pools of clustering bugs ($n = 76$) and those from the outside of the nest ($n = 174$) had 23.7 and 19.0% positive for cytopathic BCRV, respectively, and 10.5 and 4.6% positive for noncytopathic viral RNA, respectively. These differences were not significant ($\chi^2_2 = 4.3$, $P = 0.12$).

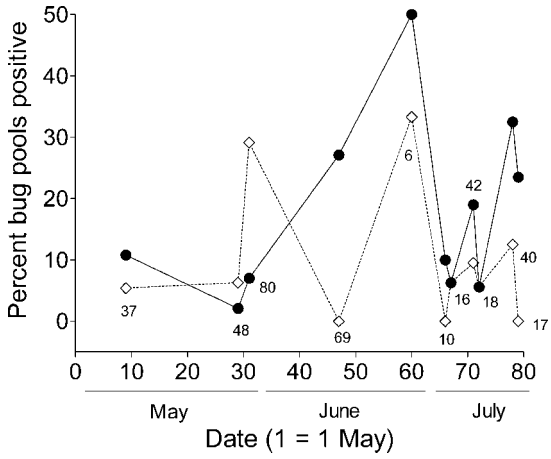

Fig. 3. Percentage of swallow bug pools positive for cytopathic BCRV (●) and noncytopathic viral RNA (◇) across all sites in relation to date of collection (1, 1 May) in summer 2004. There was no significant correlation between date and the percentage of pools positive for cytopathic BCRV (r_s = 0.25, P = 0.45, n = 11 dates) or the percentage of pools positive for noncytopathic viral RNA (r_s = −0.07, P = 0.84, n = 11). Numbers by symbols show the total number of pools collected on each date.

Effect of Date. Bugs were sampled on 11 dates between 9 May and 18 July 2004. When data were pooled across all colonies, we found no significant correlation between sampling date and the percentage of bug pools positive for either cytopathic BCRV or noncytopathic viral RNA (Fig. 3). However, for inactive colonies (sampled only in May), pools taken on 9 May (n = 22) had 18.2 and 9.1% positive for cytopathic virus and noncytopathic viral RNA, respectively, compared with pools taken on 29–31 May (n = 78) that had 6.4 and 30.8% positive for cytopathic BCRV and noncytopathic viral RNA, respectively. The differences between the sampling dates were significant (χ^2_2 = 6.0, P = 0.049). For active colonies (sampled throughout the summer), pools taken in May (n = 31) had 3.2 and 9.7% positive for cytopathic virus and noncytopathic viral RNA, respectively, compared with pools taken in June and July (n = 219) that had 22.8 and 5.9% positive for cytopathic BCRV and noncytopathic viral RNA, respectively. The differences between the months of sampling were significant (χ^2_2 = 6.6, P = 0.036). For pools taken in June (n = 76) versus July (n = 143) at active colonies, 28.9 and 2.6% in June were positive for cytopathic virus and noncytopathic viral RNA, respectively, compared with 19.6 and 7.7% in July positive for cytopathic BCRV and noncytopathic viral RNA, respectively. These differences were not significant (χ^2_2 = 4.2, P = 0.12).

Discussion

Our results indicate that whether a cliff swallow colony site is occupied in a given year, whether it is a first-time site, and whether a given nest within a colony is used by birds all influence the prevalence of cytopathic Buggy Creek virus in swallow bugs. Bug behavior and sampling date also seem associated with virus prevalence. Our results confirm previous work (from other years) showing an effect of colony size (Brown et al. 2001) on the likelihood of bugs being infected with BCRV. Finally, we found ecological differences associated with the presence of cytopathic BCRV versus noncytopathic viral RNA in bugs.

Cytopathic versus Noncytopathic BCRV. We found that 11% of swallow bug pools screened from summer 2004 contained noncytopathic BCRV RNA. These pools had no plaque growth on Vero cells, which prevented passage to increase titers for further study. However, they exhibited strong RT-PCR bands and each confirmed twice using a 208-bp region of the E2 viral genome. Previous workers have interpreted such results to arise from the presumed reduced sensitivity of the Vero cell assay (Kramer et al. 2002, Lambert et al. 2003), in which samples with lower virus titers are less likely to show cytopathicity. However, in our study some of the noncytopathic bug pools had higher viral RNA concentrations than cytopathic pools, as measured by real-time quantitative RT-PCR (A.T.M. et al., unpublished data). Thus, whether these samples exhibited plaque growth on Vero cells seemed to be unrelated to the viral RNA titers they contained, and consequently the extent of cytopathicity cannot apparently be explained by the amount of virus present in a sample. In the related western equine encephalitis virus, chronically infected vertebrate hosts (birds) and mosquitoes are routinely positive for virus by RT-PCR but often not by plaque assay (Reisen et al. 2001, 2003, Kramer et al. 2002).

The results reported here show clear ecological differences between samples with Vero-cell-cytopathic and -noncytopathic virus. Noncytopathic samples showed no correlation with colony size during the current year, in contrast to cytopathic samples (Brown et al. 2001, this study), but a strong direct relationship with colony size the previous year. Noncytopathic viral RNA was more common in pools at inactive colonies and at inactive nests within colonies, and was more likely to be found in clustering bugs (ones without recent blood meals) than in those elsewhere on a nest. In addition, at inactive sites the prevalence of cytopathic virus declined during the month of May, while the percentage of pools with noncytopathic viral RNA increased during that time.

All of these results are consistent with noncytopathic samples representing bugs that were infected up to a year or more before sampling and thus essentially constituting a historical record of infection patterns in earlier years. Swallow bugs are thought to exhibit chronic infections, apparently maintaining BCRV over the winter months (Hayes et al. 1977). The bug pools with relatively high viral RNA titers that showed no plaque formation may reflect in part seasonal changes in environmental conditions (e.g., temperature) or bug metabolic responses to host absence (A.T.M. et al., unpublished data). Regardless of the mechanisms creating noncytopathicity on Vero cells, the principal ecological issue raised by this work is

whether noncytopathic (noninfectious) BCRV, maintained apparently in a chronic state in bugs, is able to later infect birds or other bugs (perhaps after the vectors carrying it secure a bloodmeal) and thus sustain the transmission cycle at sites (e.g., inactive colonies) where it occurs. Similar questions have been posed for western equine encephalitis virus maintained in birds over the winter (Reisen et al. 2001). If the noncytopathic bug samples we identified with RT-PCR represent virus that is capable of becoming cytopathic (and thus infectious) in bug vectors at some point, the overall percentage of infected vectors was almost 27% of pools in 2004 in our study area, a relatively high vector infection rate.

BCRV and Bird Occupancy of Sites. The higher percentage of cytopathic BCRV in active swallow colonies, as opposed to sites without birds at the time of sampling, suggests an important role for cliff swallows in the seasonal amplification of this virus. This is further suggested by nests that either were active or had recently been active having much higher percentages of cytopathic bug pools than in those taken from inactive nests at a colony site, and by the seasonal increase in the percentage of cytopathic pools (from May to June–July) at sites where birds were present. Cliff swallows themselves may serve as amplifying hosts, if they maintain BCRV viremias of sufficient titers to infect bugs that feed on them. Alternatively, the presence of birds and the blood meals they provide to bugs may facilitate the propagation of virus in the bug vector and perhaps even activation of noncytopathic virus in bugs residing in and near active nests. No data are available to distinguish among these possibilities, but it is clear that active swallow colonies in a given season should be considered the foci for BCRV occurrence.

However, we did find cytopathic virus in inactive colonies: 9% of pools at unused sites exhibited BCRV that was detectable by plaque assay. These samples all came from two colony sites that had been active the preceding summer, and both had been fairly large in 2003 (360 and 400 active nests). No house sparrows, which can serve as hosts for BCRV (Hayes et al. 1977, Scott et al. 1984, Hopla et al. 1993), were present at either site in 2003 or 2004. In addition, we found cytopathic virus at sites unused by cliff swallows at the time of collection but that became active later that year. In these cases, the most likely scenario is that some infected bugs from the previous summer maintained virus over the winter and remained cytopathic the following summer. Swallow bugs (which spend the winter in swallow nests) have been hypothesized to serve as overwintering reservoirs for BCRV (Hayes et al. 1977, Brown et al. 2001), and these observations support that possibility. Cytopathic virus also was found in bugs from inactive nests within active colonies, although this is weaker evidence for virus overwintering, as infected bugs could have moved (along the substrate) into inactive nests from active nests before collection. Because cytopathic virus apparently became less common at inactive sites as the summer advanced (from early to late May), whether bugs can maintain active virus in the absence of birds for longer than a year is an interesting, unresolved question.

We found no BCRV (either cytopathic or noncytopathic) at two colony sites that had been used by cliff swallows only once and relatively little virus at a site that was in its second year of use. This could not necessarily be explained by low total numbers of bugs at these sites, because large numbers of bugs typically occur in swallow colonies even the first year (Brown and Brown 1996, also see Brown and Brown 2004), and we had 46 pools of bugs from these three sites (a sample size comparable with other colonies). These data suggest the interesting possibility that either 1) birds colonizing first-time sites are a subset of the population that is less likely to be infected, or 2) bugs that immigrate into new sites are less likely to carry BCRV. In support of the latter hypothesis, preliminary data show very low prevalence of BCRV (detectable by RT-PCR) in bugs known to be immigrants into colonies (A.T.M. and C.R.B., unpublished data). Why either of these scenarios would occur is unclear at present, although the subject of our current work. Regardless, it does seem that BCRV is less likely to be found at newly established cliff swallow colonies.

We found a relatively high percentage of pools positive for cytopathic BCRV in swallow nests that had been active earlier in the summer: almost 2 times as high as in currently active nests and more than 5 times as high as in inactive nests. Although some of these formerly active nests were nests where the nestlings had fledged before our collecting bugs, most were nests where the eggs had hatched, but the nestlings had died before fledging and the parents abandoned them. The much higher prevalence of cytopathic BCRV in the samples taken from these nests (as opposed to currently active nests) suggests that the virus might have contributed to the birds' nest failures, and/or that the seasonal cycle of virus propagation peaks at about the time of fledging. We cannot distinguish between these possibilities without better data on exact fates of nestlings that disappeared and without virus testing of dead nestlings, but the data are suggestive of a cost to cliff swallows of occupying nests containing bugs with relatively high levels of BCRV.

Bugs that cluster at nest entrances are typically adults that are seeking to disperse from an inactive nest (Brown and Brown 1996). Consequently, few of these individuals have had recent bloodmeals, in contrast to those bugs collected from the other parts of (mostly) active nests, which are often engorged with bird blood. Thus, bug behavior is associated with feeding status. That clustering, unfed bugs had a slightly lower prevalence of cytopathic BCRV than those on the outside of nests supports the possibility that having had a bloodmeal may increase the likelihood of a bug showing BCRV, either because feeding somehow activates virus in the bug or because the bloodmeal itself may consist of infected bird blood.

Comparison with Past Work. This study from the 2004 season confirms results from 1998 to 2000 in the same study area that showed a significant effect of cliff swallow colony size on the extent of cytopathic BCRV

at a site (Brown et al. 2001). Because swallow colony size is directly correlated with bug population size, we suspect that the higher rates of BCRV in larger colonies are related in part to the larger bug populations there and the greater probability of annual use by birds, both of which reduce the likelihood of bugs (and the virus they carry) going locally extinct and having to be reintroduced by birds in a given season (Brown et al. 2001). The results reported here showing little to no BCRV in bug pools from newly founded colonies and from one that had been used in only one previous year support the hypothesis that frequent colony-site use by birds is a major determinant of BCRV infection rates in bugs. Some cliff swallow colony sites in our study area are used by swallows perennially and others erratically and only rarely (Brown and Brown 1996); it now seems that the regularly used sites will be more likely to sustain BCRV during its enzootic cycles.

Studies of BCRV in southwestern Nebraska have revealed that swallow bugs are common vectors for this virus and that vector infection rates can be correlated with a number of ecological variables (Brown et al. 2001; this study). However, many aspects of this system remain unknown, and perhaps the most interesting unresolved issue is the extent to which this arbovirus successfully overwinters in bugs in the harsh winters of Nebraska and reinfects hosts and vectors in following summers.

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