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I am submitting herewith a dissertation written by Amy S. Turmelle entitled “Ecological and evolutionary factors affecting rabies virus infection in colonial insectivorous bats.” I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Ecology and Evolutionary Biology.

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Ecological and evolutionary factors affecting rabies virus infection in colonial insectivorous bats

A Dissertation Presented for
the Doctor of Philosophy
Degree
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Amy S. Turmelle
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ABSTRACT

An introductory section provides a review for the role of bats as reservoirs of infectious diseases, and highlights the rationale for investigations of host life history, ecology, and evolution in regard to bat epizootiology. Chapter 1 presents field investigations of life history, ecology, body condition, and rabies virus neutralizing antibody seroprevalence in six natural colonies of Brazilian free-tailed bats from caves and bridge roosts in Texas. Chapter 2 presents a replicate field investigation of life history, ecology, body condition, and rabies virus neutralizing antibody seroprevalence in six natural colonies of Brazilian free tailed bats from bridges and bat house roosts in Florida and Georgia. Chapter 3 evaluates the relative influence of local and landscape factors on life history, ecology, body condition and rabies virus neutralizing antibody seroprevalence in Brazilian free-tailed bats in the southern United States. Chapter 4 describes the role of host population genetic structure in big brown bat rabies virus epizootiology, and describes comparative pathogenicity of two big brown bat rabies virus isolates across several captive experimental infection studies. The information presented has been used in the development of individual, population, and metapopulation models of rabies virus epizootiology in bats.

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INTRODUCTION

Bats and emerging infectious diseases: a need for ecological models

Synopsis and Objectives

Bats (O. Chiroptera), which comprise nearly 1200 species, have been linked as natural reservoir hosts to a number of recent emerging infectious diseases (EIDs) (Calisher et al., 2006), including Ebola (Leroy et al., 2005) and Marburg (Swanepoel et al., 2007) filoviruses, SARS-like (Li et al., 2005) and related coronaviruses (Dominguez et al., 2007; Gloza-Rausch et al., 2008; Tong et al., 2009), as well as Nipah (Chua et al., 2002; Johara et al., 2001), Hendra (Halpin et al., 1996), and perhaps other novel paramyxoviruses (Chua et al., 2001; Philbey et al., 1998). Furthermore, bats are suspected reservoirs to 10 of 11 known lyssaviruses, of which rabies virus (RABV) is the best known. New World bats have long been recognized reservoirs to rabies virus (RABV) (Constantine, 1967b), and continue to be implicated in indigenous cases of human RABV infection in the Americas (Messenger et al., 2002). Historically, bats were suspected reservoirs to Lagos Bat Virus (LBV) in Africa (Boulger and Porterfield, 1958; Shope et al., 1970), although surveillance from the past two decades has revealed a wider prevalence of LBV infection among African bats than previously recognized (Kuzmin et al., 2008). Duvenhage Virus (DUVV) is also historically associated with bats in Africa, although documented human and animal infections have been rare (Nel and Rupprecht, 2007). Recently, bats were identified as potential reservoirs to several newly discovered lyssaviruses, including European Bat Lyssaviruses (EBLV-1, EBLV-2)

(Lumio et al., 1986; Mollgard, 1985), Australian Bat Lyssavirus (ABLV) (Fraser et al., 1996), Aravan Virus (ARAV) (Kuzmin et al., 1992), Khujand Virus (KHUV) (Kuzmin et al., 2003), West Caucasian Bat Virus (WCBV) and Irkut Viruses (IRKV) (Botvinkin et al., 2003). The genus *Lyssavirus* was previously classified into two major phylogroups, with suggested differences in pathogenicity between the phylogroups (Badrane et al., 2001). However, studies including the newly discovered isolates have determined that WCBV is basal to all other lyssaviruses, and does not align within either of the two main phylogroups (Kuzmin et al., 2005; Kuzmin et al., 2006). Furthermore, viruses in either of the traditional two phylogroups are known to cause classical rabies infection in humans and animals, and should be treated with equal precaution from a public health perspective (Rupprecht et al., 2002).

Many hypotheses exist for the association of bats and EIDs. These include high species diversity, long life spans, capacity for long-distance dispersal, and dense roosting aggregations (Calisher et al., 2006). IUCN reports that nearly one quarter of bat species are currently near threatened, threatened, or extinct (IUCN et al., 2008). Habitat loss is the most commonly cited factor affecting threat status among mammals, and may also contribute to disease susceptibility (Lyles and Dobson, 1993; Messenger et al., 2003a). Alternatively, theory predicts that smaller and more fragmented populations should lead to higher localized extinction of hosts and parasites (Anderson and May, 1986). In a recent comparative study across bats, threat status and population structure were significant predictors of viral richness, but geographic distribution, range size, phylogeny, body mass and average colony size were not associated with viral richness (Turmelle and Olival, In Revision). However, viral

richness was also significantly correlated with sampling effort across bat species (Turmelle and Olival, In Revision), and suggests that the process of pathogen discovery in bats, and our understanding of factors promoting viral richness, may be far from complete.

Few predictive models of bat rabies epizootiology exist, but these models have contributed to our understanding how heterogeneities within and among host populations can impact infection dynamics from individual to population level scales (Dimitrov and Hallam, 2009; Dimitrov et al., 2008; Dimitrov et al., 2007). Building from current models will require knowledge about spatiotemporal heterogeneities in the inter- and intraspecific interactions of hosts, and the impact of altered landscapes and climate, to improve epizootiological models for bat reservoirs. Given the historical relationship between bats and lyssaviruses, this system may guide our general understanding about EIDs and viral persistence in bats. My research investigates the significance of life history, ecological variation, and host phylogeography, for RABV epizootiology in two common colonial insectivorous bats in North America.

The two focal species, Brazilian free-tailed bats (*Tadarida brasiliensis*) and big brown bats (*Eptesicus fuscus*), are both widely distributed in North America but exhibit contrasting population genetic structure (Russell et al., 2005; Turmelle et al., *In Prep.*). Both species are important reservoirs of RABV in North America, are known to use a variety of natural and man-made roosts, and occasionally aggregate in large colonies, with Brazilian free-tailed bats forming the largest aggregations of any mammal (McCracken, 2003). Despite relatively high contact of both species with the public, as demonstrated by passive surveillance submission records (Mondul et al., 2003), genetic

typing of indigenous human cases of RABV infection in the United States (US) since 1980 has frequently implicated Brazilian free-tailed bat RABV (21%, 8 of 39), but not big brown bat RABV (3%, 1 of 39) (Blanton et al., 2008; Messenger et al., 2002). This dissertation highlights potential predictors of host susceptibility to RABV infection among natural colonies of Brazilian free-tailed bats across the US, and presents experimental evidence linking host phylogeography with differences in pathogenicity between RABV isolates from regional host populations. Whereas Brazilian free-tailed bats were the focal species of the field studies, big brown bats were chosen for experimental studies due to sparse information on pathogenesis of RABV in this key host, and because this species is easier to maintain for captive experimental purposes. Through the field and experimental approach, this dissertation attempts to link bat virus neutralizing antibody (VNA) response to experimental RABV infection with variation in RABV infection pressure in natural colonies, as estimated through longitudinal VNA seroprevalence data.

Chapter 1

Ecology of rabies virus exposure in colonies of Brazilian free-tailed bats (*Tadarida brasiliensis*) at natural and man-made roosts in Texas*

*This chapter is slightly modified from a paper in press:

Turmelle, AS, Allen, LC, Jackson, FR, Kunz, TH, et al. In Press. Ecology of rabies virus exposure in colonies of Brazilian free-tailed bats at natural and man-made roosts in Texas. *Vector Borne and Zoonotic Diseases*.

Introduction

Rabies virus (RABV) infection was first reported in Brazilian free-tailed bats (*Tadarida brasiliensis*) in 1954 in California and Texas (Enright et al., 1955; Sullivan et al., 1954), although the virus was probably widely circulating in several species of bats in the United States (US) long before its detection (Hughes et al., 2005). The initial detection of RABV infection in bats prompted multiple surveillance studies, and RABV-infected Brazilian free-tailed bats were detected across their geographic range in the southern US (Burns et al., 1956a; Burns and Farinacci, 1955; Burns et al., 1956b; Dean et al., 1960; Glass, 1959; Maddy et al., 1958; Richardson et al., 1966; Schneider et al., 1957). Die-offs of several thousand Brazilian free-tailed bats in New Mexico (1955, 1956) and Texas (1955) prompted additional surveillance of Brazilian free-tailed bat maternity colonies in the southwestern US, although RABV infection was confirmed in low proportions of moribund bats collected during periods of massive mortality (Constantine et al., 1968). Systematic surveillance of apparently healthy adult Brazilian free-tailed bats from maternity colonies in New Mexico has documented variable levels of RABV exposure (12-80%), and low levels of central nervous system (CNS) infection

(< 1%) (Constantine et al., 1968; Steece and Altenbach, 1989), yet few studies have examined the effects of ecological and geographic variation on the dynamics of RABV infection in bats.

Brazilian free-tailed bats aggregate annually in colonies of tens to millions of bats across their geographic range in the southwestern US (Betke et al., 2008; McCracken, 2003) and provide substantial ecosystem and economic services to agricultural regions of south-central Texas (Cleveland et al., 2006; Federico et al., 2008). The largest aggregations of these bats often function as maternity colonies, whose diets are supported by the emergence of insect prey from agricultural croplands in south-central Texas (e.g. corn, cotton) during the summer months (Kunz et al., 1995; Lee and McCracken, 2005). This species is known to colonize a variety of roosts, including natural (e.g. caves, trees) and man-made structures (e.g. mines, bridges, bat houses, buildings). In Texas, large colonies occur in caves (Betke et al., 2008; Davis et al., 1962; McCracken, 2003) and, increasingly, in the expansion joints of highway bridges (Keeley and Keeley, 2004; Keeley and Tuttle, 1999), with smaller colonies found in buildings (Davis et al., 1962; Scales and Wilkins, 2007). Texas bridge colonies are often associated with heavy vehicular or rail traffic, which contributes noise, air, and ground pollution to the local environment. Little is known about how these novel stimuli affect the immune competence and epizootiology of bats living in man-made roosts.

This study investigates ecological and geographic effects on rabies viral neutralizing antibody (VNA) prevalence in six colonies of Brazilian free-tailed bats in south-central Texas. Periods of sampling corresponded with seasonal changes in reproductive activity of adult female bats (e.g. pregnancy, lactation, and non-

reproductive periods). We predicted that RABV exposure would increase following synchronized parturition in this species, when the overall population size, contact rates, and the proportion of susceptible bats are expected to increase. We expected lower immune competence from bats in bridge roosts due to the perceived stresses associated with anthropogenic disturbances, potentially resulting in fewer bats mounting immune responses to RABV exposure, and lower VNA seroprevalence (Smith, 1981; Smith et al., 1982). We expected periodic fluctuations in RABV exposure to be greater in adult females, compared to adult males, as reproductive females presumably have greater contact with clusters of susceptible young. As sex ratios are more heavily skewed in favoring females at cave roosts, we expected periodic fluctuations in RABV exposure to be greater in cave roosts. For juveniles, we expected higher RABV exposure during early periods of sampling following parturition, as previous data have suggested high levels of RABV infection during early weeks of life (Constantine, 1986; Steece and Altenbach, 1989).

Methods

Animal Sampling

All capture and handling procedures were approved by the University of Tennessee Institutional Animal Care and Use Committee, and comply with the American Society of Mammalogists guidelines for the use of wild mammals in research (Gannon et al., 2007). Field studies were conducted under Texas Parks and Wildlife permit #SPR-0305-058. All persons involved with the capture and handling of bats

received RABV pre-exposure prophylaxis (Manning et al., 2008), and appropriate personal protective equipment was worn during sampling.

A total of six sites in south-central Texas were sampled between May and October 2005, including three caves and three bridges: Davis Blowout Cave (DBC), Frio Cave (FC), Eckert James River Cave (JRC), McNeil Bridge (MB), Seco Creek Bridge (SCB), and East Elm Creek Bridge (EEB) (Figure 1.1). Free-ranging bats were captured at emergence from all sites between 18:00 and 21:00, using a combination of harp trap and hand nets, and all bats were immediately freed from traps or nets and placed into individual cloth bags (Kunz and Kurta, 1988). Standard measurements were taken on all bats, including mass, right forearm length, age (Anthony, 1988), sex, and reproductive condition (Racey, 1988). A sample (80-100 µl) of whole blood was collected in sterile heparinized microcapillary tubes following aseptic preparation and puncture of a peripheral wing vein (Kunz and Nagy, 1988). Plasma was separated within two hours of blood draw, and stored at -20°C. Bats were observed for clinical signs of RABV infection during processing (i.e. odd vocalizations, ataxia, paresis, paralysis). Before release at the site of capture, a non-toxic ink tattoo was applied to all bats to prevent re-sampling of individuals and to assess site fidelity through the sampling season. An index of body condition was calculated by taking the ratio of body mass (g) to length of the right forearm (mm).

All six colonies were sampled periodically for a minimum of three out of (Early, Mid, Late) four stages of the female bats' seasonal life history: Early – May through mid-June (pregnancy), Mid – mid-June through July (lactation), Late – August (non-reproductive), and Pre-migratory – September through October (Figure 1.2). Mating

activity has been observed in March and early April at MB (Keeley and Keeley, 2004), and post-copulatory vaginal plugs were found in females from DC in March but not April (Davis et al., 1962). This study began in early May and males did not show evidence of reproductive activity (i.e. descended testes) during the periods sampled. In the Early period, colonies are still assembling as individuals arrive from Mexico at maternity sites that are primarily comprised of adult females in the early to middle stages of gestation. The Mid reproductive period immediately follows synchronized parturition, which occurs in early June (Constantine, 1967a; Davis et al., 1962; McCracken and Gustin, 1991). The majority of adult female bats are nursing pups during this period, when milk was easily expressed from nursing females that have visibly swollen nipples that are devoid of hair. Colony sizes peak and are most stable during the Mid reproductive period (Betke et al., 2008; Constantine, 1967a; Davis et al., 1962), as pups and juvenile bats remain in the roost while adults are foraging, and switching roosts with young may be risky and energetically costly for females. In the Late period, most adult females are post-lactational (non-reproductive), and juveniles achieve adult size and forage independently. Post-lactation adult females are characterized by the growth of new hair around the nipple and no milk expression following palpation. During the pre-migratory phase, all adult bats and young-of-the-year are considered non-reproductive, and are preparing for autumn migration to Mexico (Figure 1.2).

The timing and patterns of reproductive activity in adult female bats observed during this study are consistent with previously published accounts (Constantine, 1967a; Davis et al., 1962; McCracken and Gustin, 1991). The data suggest that, despite greater proportions of male bats in bridge colonies, reproductive schedules and

activity patterns for adult female bats are similar in cave and bridge colonies (Figure 1.2).

Detection of RABV neutralizing antibodies

A modified rapid fluorescent focus inhibition test (RFFIT) (Jackson et al., 2008; Smith et al., 1996), using rabies challenge virus standard (CVS-11, V399) (Briggs et al., 1998), was used to assay for RABV-specific viral neutralizing antibodies (VNA) in the blood plasma of individual bats. The lowest bat plasma dilution tested in the RFFIT assay was 1:4, and sequential 2-fold dilutions were tested up to 1:2048. Rabies VNA endpoint titers of individual bats were calculated (Reed and Muench, 1938), and were converted to international units (IU/ml) by comparison to a control standard rabies immune globulin (SRIG) containing 2 IU/ml. Final titers of less than 0.06 IU/ml were considered negative for rabies VNA. Positive VNA titers (≥0.06 IU/ml) were interpreted as being indicative of prior RABV exposure. The choice of this cutoff value follows previous studies for lyssavirus surveillance using bat and non-bat sera (Blanton et al., 2007; Jackson et al., 2008; Lumlertdacha et al., 2005; Rupprecht et al., 2005; Shankar et al., 2004). A previous study has demonstrated that the immunoglobulin G (IgG) fraction of the bat serum is responsible for neutralization activity against RABV (Shankar et al., 2004).

Statistical Analyses

Rabies VNA seroprevalence was treated as a binomial response variable for all analyses. For statistical analyses, the seroprevalence data were partitioned into two cohorts: adults (N=463), and juveniles (N=50). For statistical analyses on the adult cohorts, the Pre-migratory period was excluded owing to uniformly low sample sizes and uneven sampling across sites. For the juvenile cohort, analyses were conducted with data obtained across six sites during the Late and Pre-migratory period.

A series of hierarchical logistic models were tested using SAS v.9.1 (SAS Institute, Inc., Cary, NC) to investigate significant ecological predictors of rabies VNA seroprevalence. The central question focused on testing for effects of roost type on rabies VNA seroprevalence, particularly in maternity colonies of reproductively active adult female bats. A nested mixed logistic model (PROC GLIMMIX) was used to control for variation among sites that represent the two roost types (cave vs. bridge) sampled in this study, with site treated as a random effect nested within roost type, and fixed effects of roost type, period of sampling, sex, and reproductive condition (α=0.05). In the absence of significant effects of roost type, models were simplified to non-nested mixed logistic models, with site still treated as a random effect (α=0.05). In the nested and non-nested models, individual body condition was tested as a covariate. All two and three-way fixed effect and covariate interactions were tested (α=0.05). Non-significant covariate-fixed effect interactions were removed from the model prior to testing fixed effects (Engqvist, 2005). Marginally predictive ($0.05<\alpha<0.10$) fixed effect interactions

were retained in the models. For models with significant fixed effects, pair-wise contrasts among all levels of the fixed effect were tested (α=0.05).

Although tested as a covariate in the seroprevalence models, body condition data were also analyzed separately for Early, Mid, and Late period in male and female adult cohorts, and during the Late and Pre-migratory period for the juvenile cohort, to investigate roost type, gender, and seasonal effects. A nested mixed ANOVA model was used to test for differences in body condition by roost type (α=0.05). All nested, fixed, and random predictors were treated as described above. In the absence of significant roost type effects, models were simplified to non-nested mixed ANOVA models. Tukey's post-hoc means separation test was used to compare all pair-wise levels for significant fixed effects (α=0.05).

Model testing was also conducted with the adult and juvenile body condition and rabies VNA seroprevalence data sets. Akaike Information Criterion (AIC) values were used to rank all possible models (Burnham and Anderson, 2002), and determine the optimal set of predictors to explain the data.

Results

A total of 615 Brazilian free-tailed bats were sampled for rabies VNA between May and October 2005. None of the bats presented clinical signs of RABV infection during handling and sampling procedures. None of the bats were re-captured during the sampling season. Seasonal and ecological predictors of VNA seroprevalence were investigated in separate cohorts of adult (n=463, Table 1.1), and juvenile bats (n=50, Table 1.2)

VNA Seroprevalence - Adults

In the nested mixed logistic model of VNA seroprevalence among adult bats (no. sites=6, n=463, Table 1.3), there was a significant interaction of roost type and period (**df=2, 449, F=3.03, p=0.05**; site [roost]=0.31 [±0.26]) (Figure 1.3). Contrasts on the roost by period interaction did not reveal significant differences between roosts for any period. Data were subdivided by roost type for additional testing of period and gender effects in non-nested models.

In the non-nested logistic model of VNA seroprevalence for adult bats in cave colonies (no. sites=3, n=232; Table 1.4), period was the only significant predictor in the model (**df=2,224, F=3.66, p=0.03**; site=0.47 [±0.55]). Contrasts for the levels of period indicate that VNA seroprevalence during the Mid period is significantly greater than during the Early (p=0.006) or Late (p=0.03) periods in cave colonies.

In the non-nested logistic model of VNA seroprevalence for adult bats in bridge colonies (no. sites=3, n=231; Table 1.5), none of the fixed effects in the model were significant predictors of VNA seroprevalence (**p>0.1**; site=0.15 [±0.21]).

VNA Seroprevalence - Juveniles

Limited sampling prevented the testing of interactions between fixed effects in nested and non-nested models of VNA seroprevalence for juvenile bats (sites=6, n=50; Table 1.6). In the nested model, roost effect was not a significant predictor of VNA seroprevalence (**df=1, 4, F=0.99, p=0.38**; site [roost]=0.24 [±0.73]) (Figure 1.4). In the

non-nested model among juvenile bats, period was not a significant predictor of variation in VNA seroprevalence (**df=1, 42, F=1.67, p=0.20**), but body condition was a marginally predictive covariate of VNA seroprevalence (**df=1, 42, F=3.08, p=0.09**; site=0.14 [±0.54]). When controlling for the effects of period, lighter juvenile bats were more likely to be seropositive.

In the nested mixed logistic model comparing VNA seroprevalence among adult and juvenile bats during the Late period (n=138), age was not a significant predictor of VNA seroprevalence (**df=1, 131, F=0.45, p=0.50**; site [roost]=0.93 [±1.07]) (Table 1.7).

Body Condition – Adults

The nested mixed ANOVA model comparing adult bats in Texas during the Early, Mid, and Late periods (sites=6, n=519; Table 1.8) explained 27% of the variation in body condition, and a significant three-way interaction was detected between gender, roost type, and period (**df=2, 505, F=5.25, p=0.006**; site [roost] z=0.75, p=0.23). Data were subset by gender for subsequent analyses, as female body condition is likely to be highly sensitive to changing reproductive status, whereas males are non-reproductive during the sampling season.

The nested mixed ANOVA model comparing adult female bats (no. sites=6, n=331; Table 1.8) explained 46% of the variation in body condition, and a marginally significant interaction was detected between roost type and reproductive status (**df=2, 325, F=2.90, p=0.06**; site [roost] z=0.86, p=0.19). Roost type (**df=1, 5, F=9.53, p=0.03**) and reproductive status (**df=2, 325, F=30.7, p<0.0001**) were significant predictors of adult female body condition, and contrasts indicate that the body condition of female

bats from cave colonies is significantly lower compared to females from bridge colonies only during the lactation phase. Alternatively, when controlling for roost type, pregnant females were heavier, and body condition decreased in females that were lactating or non-reproductive (Figure 1.5).

The nested mixed ANOVA model comparing adult male bats (no. sites=4, n=158; Table 1.8) explained 11% of the variation in body condition, and a significant roost type by period interaction was detected (**df=2, 150, F=3.71, p=0.03**; site [roost] z=0.82, p=0.21). Contrasts indicate that body condition is only variable among male bats from the cave colony; however, comparisons between males from cave and bridge colonies were not significantly different for any period (Figure 1.6).

Body Condition – Juveniles

The nested mixed ANOVA model comparing juvenile bats across the Late and Pre-migratory periods (no. sites=6, n=67; Table 1.9) explained 49% of the variation in body condition, and period was the only significant predictor in the model (**df=1, 52, F=13.68, p=0.0005**; site [roost] z=0.95, p=0.17). Contrasts on the levels of period indicate that juvenile body condition is significantly higher during the Pre-Migratory period compared to the Late period (Figure 1.7).

A nested mixed ANOVA model was used to test for age effects on body size (i.e. right forearm length) and body condition among adult and juvenile cohorts during the Late period (n=179). Highly significant age effects were detected in body condition among adult and juvenile bats (**df=1, 163, F=126.5, p<0.0001**; site [roost] z=0.43,

p=0.33), although adults had marginally larger body size compared to juvenile cohorts (**df=1, 175, F=2.89, p=0.09**; site [roost]=0) (Table 1.10).

Comparisons of model AIC values for VNA seroprevalence and body condition data sets converged on identical sets of significant predictors when compared to the statistical tests for partitioned data (Table 1.11 – Table 1.14). The best models of VNA seroprevalence in adult bats did not include body condition as a covariate, but the best models of VNA seroprevalence in juveniles did include this covariate (Table 1.11, Table 1.12).

Discussion

The exposure of Brazilian free-tailed bats to RABV, as evidenced by VNA seroprevalence, is highly variable, but the patterns of exposure indicated by this study are consistent with previous research for this species. Rabies VNA seroprevalence of colonies varied from low levels of exposure (0-5%) to extremely high levels (>50%) among sites. All colonies exhibited evidence of RABV exposure, further supporting wide geographic prevalence of infection, which is consistent with previous surveys (Constantine et al., 1968; Steece and Altenbach, 1989). Our results indicate that roosting ecology and reproductive activity are important factors affecting rabies VNA seroprevalence and nutritional status among colonies of bats.

Adult Females

All of the sites sampled in this study contain seasonal colonies of bats that migrate from Mexico (Cockrum, 1969; Davis et al., 1962; Villa-R and Cockrum, 1962),

and consist primarily of adult female bats and their offspring. Contrary to our expectations, roost type alone was not a significant predictor of RABV exposure, but seasonal fluctuations in exposure were affected by roost type. In cave colonies, RABV exposure significantly increased during the Mid period, whereas RABV exposure at bridge colonies was more uniform across the time periods sampled (Figure 1.3). The increase in RABV exposure following parturition among adult females at cave colonies may be associated with heightened RABV infection in susceptible young (Constantine, 1986; Steece and Altenbach, 1989), and an increase in contact rates associated with adult females nursing within clusters of young (McCracken and Gustin, 1991). Although we did not obtain comparative measurements of the densities of bats on roost surfaces in caves and bridges, census data suggest lower colony sizes in bridges when compared to caves (Betke et al., 2008; Constantine, 1967a; Keeley and Keeley, 2004). Sex ratios of adult bats are significantly skewed at cave colonies when compared to bridge or building colonies, with caves having almost exclusively adult female bats prior to parturition (Constantine, 1967a; Davis et al., 1962; Keeley and Keeley, 2004; McCracken and Gustin, 1991). The proportional number of adult female bats is particularly important in regard to the increase in colony size following parturition; i.e. caves support larger colonies and greater proportions of adult females prior to parturition and therefore are subject to greater increases in colony size. Thus, we expect greater increase in contact rates in caves following parturition, when compared to bridge colonies. However, the largest bridge colony sampled (MB) had a population size comparable to cave colonies sampled [MB = 0.75 million; DBC, JRC, FC = 0.43-1.3 million (Betke et al., 2008; Keeley and Keeley, 2004)], but did not vary in RABV

exposure across the sample periods. We conclude that parturition, sex ratios, and colony size, contribute to the periodicity in RABV exposure detected among adult female bats in cave colonies.

When similar statistical analyses are performed on previously published data from adult female bats at Carlsbad Cavern, NM [Table 4 in (Constantine et al., 1968)], we also detect a significant increase in rabies VNA seroprevalence between May 1956, and the period following parturition (July 1956), that decreases by August 1956 ($LR\chi_2$=7.38, p=0.03). While the estimates of rabies VNA seroprevalence are not directly comparable to the estimates obtained in this study, the seasonal patterns of exposure in adult female bats at cave colonies are consistent. However, a later study at Lava Cave, NM, did not report significant fluctuation in RABV infection or VNA seroprevalence in adult female bats during the reproductive season, despite a peak of RABV infection in juveniles in the early weeks following parturition (Steece and Altenbach, 1989). The discrepancy in patterns among adult females may result from ecological variation across years and colonies, but may also reflect differences in diagnostics and survey techniques (e.g. capture in roost versus during emergence) (Constantine et al., 1968; Steece and Altenbach, 1989).

Contrary to our expectations, adult female bats roosting in bridges had higher body condition and higher VNA seroprevalence when compared to cave-roosting females during most reproductive periods (Figure 1.3, Figure 1.5). Despite the perceived stresses associated with anthropogenic disturbance at man-made roosts, bridge-roosting females may have greater energy to allocate to mounting immune defenses (e.g. rabies VNA) when compared with cave-roosting females. Alternatively,

there may be differences in RABV infection pressure between cave and bridge roosts. RABV infection prevalence was estimated at a bridge colony of Brazilian free-tailed bats in New Mexico near Carlsbad Cavern (Constantine et al., 1968). Although the infection estimates were higher in normal-appearing bats at the bridge colony, it is unclear whether bats at the two different roosts were collected in the same manner (i.e. collected by hand versus in flight). In particular, most (4 of 5) of the infected individuals at the bridge roost were immature young, and perhaps would not have been included in similar samples of bats at the cave roost [Tables 2 and 3 in (Constantine et al., 1968)]. Despite equivocal evidence comparing RABV infection between roost types, it is possible that higher VNA seroprevalence detected among bridge colonies in this study may be associated with higher RABV infection prevalence at bridge roosts. As the current study was non-destructive, there were insufficient data to test the effect of roost type on infection prevalence. Given the differences in spatial arrangements of individuals within cave roosts (i.e. radial) versus bridge roosts (i.e. linear), we presume that higher VNA seroprevalence among bridge roosting bats is not linked to higher contact rates.

As noted in earlier studies, the massive presence of hematophagous parasites, high concentrations of ammonia, and respiratory pathogens (e.g. *Histoplasma capsulatum*) that flourish in cave roosts (Constantine et al., 1968; Davis et al., 1962) may contribute to energetic trade-offs in cave roosting bats, resulting in lower body condition and generally lower rabies VNA seroprevalence, except following parturition. Our model of immune competence, as it relates to rabies VNA seroprevalence, has been adapted from RABV infection studies in mice (Smith, 1981; Smith et al., 1982).

Recent studies suggest that parasitism and immunocompetence may vary during reproduction in other colonial bat species (Christe et al., 2000; Pearce and O'Shea, 2007), and may be influenced by roosting ecology in Brazilian free-tailed bats (Allen et al., 2009). Interestingly, one study found a strong relationship between an index of adaptive immune response and colony sizes for Brazilian free-tailed bats in cave and bridge colonies in Texas (Allen et al., 2009), but extrapolation to the control of RABV infection within host populations remains circumstantial. We presume that lower immune competence leads to lower VNA seroprevalence, but lower immune competence may lead to more a productive viral infection, thus causing induction of a VNA response that may not have been elicited in an immunocompetent animal. Additional experimental studies are needed to address alternative scenarios on the effects of body condition and immune competence on the humoral (VNA) response to RABV exposure.

Adult Males

Contrary to our expectations, patterns of VNA seroprevalence among adult male bats did not differ from adult females, as gender was not a significant predictor in the nested mixed logistic model. Evidence of gender-specific differences in susceptibility to RABV infection among Brazilian free-tailed bats has been lacking. Male bats living in bridge colonies appeared to have uniform body condition throughout the sampling season, whereas the [few] males living in cave colonies exhibited lower body condition during the Early period (Figure 1.6). The data suggest that males living in cave colonies might be subject to high competition for resources when females are pregnant, but that

their body condition rebounds when females are lactating. Prey resources are known to have episodic emergences from corn crop fields during the Mid period (J. K. Westbrook, unpublished data), which may alleviate expected competition between lactating females and males. Periodicity was observed in the VNA seroprevalence among some adult male bats, and may relate to the proportion of time spent in the roost during the Mid period. Male and non-reproductive female Brazilian free-tailed bats spend significantly more time in roosts following parturition when compared to lactating adult females, consistent with lactating females having higher energetic requirements that require longer or more frequent nightly foraging bouts (Kunz et al., 1995; Lee and McCracken, 2001). Thus, although males have less direct contact with developing young compared to nursing females, they may experience similarly high RABV exposure due to significantly longer duration of time spent in the roost with young during the Mid period. Competition for resources, and related activity patterns of among classes of reproductive and non-reproductive bats, may be influenced by environmental conditions that impact the availability of prey (e.g. wetter summers lead to higher prey abundance), in terms of energetic trade-offs between foraging behavior and predator avoidance. One study has suggested that nutritional status, and immune competence, may be strongly tied to variation in prey availability across years in greater-mouse eared bats (*Myotis myotis*) (Christe et al., 2000). Despite the importance of adaptive immune response in control of RABV infection (Hooper et al., 1998), the field immune assays that are currently used with bats have not been validated with experimental infections, and preclude direct inferences for host resistance to RABV infection in wild bats. Interestingly, periodicity in rabies VNA seroprevalence was not detected in adult male

bats at Carlsbad Cavern, NM, between May-August of 1956 (p=0.91) [Table 4 in (Constantine et al., 1968)], although comparisons of environmental conditions and prey availability between these studies were not possible. The impact of prey availability on the activity budgets of different classes of reproductive and non-reproductive bats warrants additional study for estimating contact rates and disease exposure in colonial species.

Juveniles

After parturition in early June, pups become densely clustered on the ceiling and walls of caves (McCracken and Gustin, 1991), and in expansion joints at bridge roosts (personal observations). At four weeks post-partum, the density of roosting clusters may decrease, although clusters often remain separate from adult groups up to six weeks post-partum (McCracken and Gustin, 1991). Although young pups were not observed in the crevices of two of the bridge sites (SCB, EEB), juvenile bats were captured in flight during emergence from all colonies sampled. Our capture methods resulted in sampling of juveniles that were likely to be four weeks of age or older, and were engaging in practice flights or emerging to forage independently. Juvenile bats with rabies VNA were detected from all colonies sampled except JRC, but samples sizes at JRC were low (n=3) (Table 1.2). We did not find evidence for significant variation in juvenile RABV exposure by roost type or period, but do find a marginal association with body condition, and juvenile bats with lower body condition were more likely to be rabies VNA seropositive. Without more robust and even sampling, we cannot conclude that there is a significant association of RABV exposure and juvenile

body condition. Although separate analyses of body condition detected increasing values from Late to Pre-migratory periods (Figure 1.7), the body condition of juvenile bats is expected to steadily increase following parturition, initially due to rapid development during lactation (Kunz and Robson, 1995) and later as both adult and young of the year are preparing for the fall migration.

RABV infection in 19% (76 of 395) of 5-11 day old Brazilian free-tailed pups was reported from FC in 1974, but no infection (0 of 284) in pups less than 5 days of age, evidence that many pups may be infected shortly after birth (Constantine, 1986). Elevated levels of RABV infection were reported in juvenile bats at Lava Cave, NM, following parturition (July and August) (Steece and Altenbach, 1989). The data in this study are consistent with, but do not lend evidence to, elevated RABV infection in pups during the first four weeks following parturition (McCracken and Gustin, 1991). RABV infection among juvenile bats during the early weeks following parturition may result from contact with infected adult female bats, or aerosol RABV exposure in cave roosts (Baer and Bales, 1967; Constantine, 1967c; Constantine et al., 1972; Davis et al., 2007; Winkler, 1968). No controlled experiments have been conducted to compare the susceptibility of adult versus juvenile bats to RABV infection, nor are there controlled studies which document the significance of maternally transmitted rabies VNA in protecting pups against RABV infection. One study suggests that greater mouse-eared juvenile bats (*M. myotis*) harbor greater numbers of reproductive parasites compared to adults, perhaps due to lower immune competence (Christe et al., 2000), although another pair of studies found higher parasite intensities on adult big brown bats (*Eptesicus fuscus*) (Pearce and O'Shea, 2007), and did not report any association of

rabies VNA with parasite loads on adult or juvenile bats (Pearce et al., 2007). Experimental evidence is needed to evaluate whether pups and volant juveniles may be immunocompromised compared to adult bats, and may experience greater susceptibility to doses of RABV that produce abortive infections in immunocompetent adults (e.g. aerosol inoculation) (Baer and Bales, 1967; Davis et al., 2007), particularly with regard to the significance of maternally-acquired antibodies for resistance to RABV infection (Xiang and Ertl, 1992).

Conclusions

Seasonality has recognized importance for epizootiological processes (Altizer et al., 2006; Nelson et al., 2002). Using rabies VNA seroprevalence as an indicator of RABV exposure, infection pressure varied with seasonal changes in reproductive activity among cave, but not bridge, roosting colonies of bats. Further study is necessary to address the contact structure of bats within colonies among different roosts, to estimate additional relevant parameters of disease exposure among different cohorts, in the context of seasonal life cycles of the bats (Constantine, 1967a; Davis et al., 1962; Lee and McCracken, 2001). A study of several vespertilionid bat species found an increase in the prevalence of coronavirus infection in adult female bats during lactation (Gloza-Rausch et al., 2008), and another study found higher Hendra virus seroprevalence in pregnant and lactating female little red flying foxes (*Pteropus scapulatus*) (Plowright et al., 2008), both independently suggesting that epizootiology in colonial bats may be influenced by seasonal life history. Non-destructive long-term sampling of natural colonies is needed to provide additional insight into how roosting

and behavioral ecology affect enzootic RABV infection in colonial and solitary species, with regard to the effects of life history and environmental variation across years.

Although our understanding of how immune competence of individual bats contributes to RABV infection is in its infancy, it has been suggested that immunocompromised individuals are likely to play a strong role in the persistence of enzootic rabies in Brazilian free-tailed bats (Constantine, 1988). Individual and population level models of bat rabies have been proposed using the results obtained in this and similar studies (Dimitrov and Hallam, 2009; Dimitrov et al., 2008; Dimitrov et al., 2007), and have demonstrated that immunocompromised bats can indeed contribute significantly to infection persistence, and that the infection dynamics within colonies may lead to persistence of immunocompromised animals in the population via frequency-dependent processes. Incorporating seasonal variation into the immunotypic structure of colonies may provide additional insight into rabies infection dynamics among colonial bat reservoirs.

Chapter 2

Ecology of rabies virus exposure in colonies of Brazilian free-tailed bats (*Tadarida brasiliensis*) at bridge and bat house roosts in Florida and Georgia

Introduction

Brazilian free-tailed bats are abundant and widely distributed across the southern United States (US), where they inhabit a variety of natural and man-made roosts. Rabies virus (RABV) infection has been detected from Brazilian free-tailed bats across the US (Burns et al., 1956a; Burns and Farinacci, 1955; Burns et al., 1956b; Dean et al., 1960; Glass, 1959; Maddy et al., 1958; Richardson et al., 1966; Schneider et al., 1957), but there has been a lack of systematic surveillance studies addressing spatiotemporal trends in RABV exposure. Longitudinal studies of RABV infection exist for two cave colonies in New Mexico (NM) (Constantine et al., 1968; Steece and Altenbach, 1989), and a limited cross-sectional survey provided additional evidence of widespread infection pressure across southwestern colonies (Burns et al., 1956a). However, minimal surveillance has been conducted for bats in non-cave roosts, particularly outside of the western portion of their geographic range in the US.

Ecological variation has been documented among Brazilian free-tailed bat populations, particularly in the western US, but very little is known about the ecology of populations in the southeastern US. Historically, hollows of mangrove trees may have been typical roosts for Brazilian free-tailed bats in the southeastern US (Jennings, 1958), although use of man-made structures by this species was also historically reported (Sherman, 1937). Contemporary populations in the southeastern US are

documented only in artificial, man-made roosts (Wilkins, 1989). In Florida, caves are frequently inhabited by southeastern myotis bats (*Myotis austroriparius*) (Gore and Studenroth, 2005; Wilkins, 1989), but may not be suitable for Brazilian free-tailed bats. Whereas Brazilian free-tailed bats in the southwestern US are known to engage in long-distance seasonal migrations (Cockrum, 1969; Villa-R and Cockrum, 1962), populations may be year-round residents in the southeastern and northwestern US (Krutzsch, 1955; Lee and Marsh, 1978; Sherman, 1937). Given regional variation in ecology and behavior, patterns and predictors of RABV exposure in Brazilian free-tailed bat populations may also vary across their geographic range in the southern US.

This study investigates the effects of roost ecology and seasonal life history on rabies viral neutralizing antibody (VNA) seroprevalence and body condition for six colonies of Brazilian free-tailed bats at two types of man-made roosts in southern Georgia and Florida. Periods of sampling correspond with seasonal changes in reproductive activity of adult female bats (e.g. pregnancy, lactation, and non-reproductive periods). We predicted that RABV exposure would increase following synchronized parturition, when the overall population size, contact rates, and the proportion of susceptible bats are expected to increase. As adult females have greater contact with susceptible young compared to male bats, we expected females to have higher VNA seroprevalence than males. We expected lower immune competence from bats in bridge roosts due to the perceived stresses associated with anthropogenic disturbances, potentially resulting in fewer bats mounting VNA responses to RABV exposure (Smith, 1981; Smith et al., 1982).

Methods

Animal Sampling

All capture and handling procedures were approved by the University of Tennessee Institutional Animal Care and Use Committee, and comply with the American Society of Mammalogists guidelines for the use of wild mammals in research (Gannon et al., 2007). Field research was conducted under Georgia permit #29-WSF-05-14 and Florida permit #WX06055. All persons involved with the capture and handling of bats received RABV pre-exposure prophylaxis (Manning et al., 2008), and appropriate personal protective equipment was worn during sampling.

A total of six sites in the southeastern US (i.e. Georgia, Florida) were sampled monthly at four bridges and two bat-house colonies: Sandy Creek Bridge (SACB; FL), Tenmile Creek Bridge (TCB; FL), Black Creek Bridge (BCB; FL), Aurantia Bridge (AUB; FL), Quitman Bat House (QBH; GA) and Gainesville Bat House (GBH; FL) (Figure 2.1). Large (~10,000) and small (~200-500) bat colonies were sampled among bridge and bat-house roosts. Sampling was conducted from May to August 2006 during three life history stages: Early – May (pregnancy), Mid – June through July (lactation), and Late – August (non-reproductive) (Figure 2.2). Free-ranging bats were captured at emergence from all bridge sites between 19:00 and 21:00, using a combination of mist and hand nets, and all bats were immediately freed from nets and placed into individual cloth bags (Kunz and Kurta, 1988). At bat-house roosts (QBH, GBH), bats were captured in flight using mist and hand nets between 19:00 and 24:00, but were otherwise handled and sampled identically to bats from bridge sites. Standard measurements were taken on

all bats, including mass, right forearm length, age (Anthony, 1988), sex, and reproductive status (Racey, 1988). A sample (80-100 µl) of whole blood was collected in sterile heparinized microcapillary tubes following aseptic preparation and puncture of a peripheral wing vein (Kunz and Nagy, 1988). Plasma was separated within two hours of blood draw, and stored at -20°C. Bats were observed for clinical signs of RABV infection during processing (i.e. odd vocalizations, ataxia, paresis, paralysis). Before release at the site of capture, a non-toxic ink tattoo was applied to all bats to identify previously sampled individuals and to assess site fidelity through the sampling season. An index of body condition was calculated by taking the ratio of body mass (g) to length of the right forearm (mm).

Among southwestern colonies, mating is thought to primarily occur in lower latitudes prior to northward migration into the US, although copulation was observed in a Texas bridge colony during March and April (Keeley and Keeley, 2004), and indirect evidence (i.e. post-copulatory vaginal plugs) was found among females from a cave in Texas in March (Davis et al., 1962). Based on a study of a building colony in Gainesville, FL, it was suggested that mating among Brazilian free-tailed bats occurs during a one-week period in March, although copulations were not observed (Sherman, 1937). None of the male bats that we sampled showed evidence of mating activity (i.e. descended testes) during any of the periods sampled. During the Early period of sampling, adult females are in the early to middle stages of gestation. Synchronized parturition occurs in early June, and the majority of adult female bats were nursing pups during the Mid period of sampling in June and July, when milk was easily expressed from nursing females that have visibly swollen nipples that are devoid of hair. Colony

sizes likely peak and are most stable during June and July, as pups and juvenile bats remain in the roost while adults are foraging, and switching roosts with young may be risky and energetically costly for females. In the Late period, most adult females are post-lactational (non-reproductive), and juveniles achieve adult size and forage independently. Post-lactation adult females are characterized by the growth of new hair around the nipple and no milk expression following palpation. Thus, during the Late period sampling, most adult bats and all volant young-of-the-year are non-reproductive (Figure 2.2).

The timing and patterns of reproductive activity in adult female bats observed during this study are consistent with previously published accounts (Constantine, 1967a; Davis et al., 1962; McCracken and Gustin, 1991; Sherman, 1937). The data suggest that reproductive schedules for Brazilian free-tailed bats are similar in bridge and bat-house colonies in the southeastern US compared to colonies in Texas and New Mexico (Figure 1.2, Figure 2.2).

Detection of RABV neutralizing antibodies

A modified rapid fluorescent focus inhibition test (RFFIT) (Jackson et al., 2008; Smith et al., 1996), using rabies challenge virus standard (CVS-11, V399) (Briggs et al., 1998), was used to assay for RABV-specific viral neutralizing antibodies (VNA) in the blood plasma of individual bats. The lowest bat plasma dilution tested in the RFFIT assay was 1:4, and sequential 2-fold dilutions were tested up to 1:2048. Rabies VNA endpoint titers of individual bats were calculated (Reed and Muench, 1938), and were converted to international units (IU/ml) by comparison to a control standard rabies

immune globulin (SRIG) containing 2 IU/ml. Final titers of less than 0.06 IU/ml were considered negative for rabies VNA. Positive VNA titers (≥0.06 IU/ml) were interpreted as being indicative of prior RABV exposure. The choice of this cutoff value follows previous studies for lyssavirus surveillance using bat and non-bat sera (Blanton et al., 2007; Jackson et al., 2008; Lumlertdacha et al., 2005; Rupprecht et al., 2005; Shankar et al., 2004). A previous study has demonstrated that the immunoglobulin G (IgG) fraction of the bat serum is responsible for neutralization activity against RABV (Shankar et al., 2004).

Detection of viral RNA in saliva

Oropharyngeal samples were collected on paired sterile polyester swabs soaked in minimal essential medium (MEM-10). One swab was immediately placed in one ml of TRIzol® (Invitrogen Corporation, Carlsbad, CA), and stored at -80°C until testing, and the other swab was placed in one ml of MEM-10 for potential viral isolation. Viral RNA was extracted from the swab samples fixed in TRIzol®, and the reverse transcriptase polymerase chain reaction (RT-PCR) technique was used to attempt amplification of viral RNA from salivary samples as described previously (Jackson et al., 2008; Orciari et al., 2001). Salivary samples were tested from a subset of bats (69%, 295 of 425), mixed evenly across gender, age, and time periods.

Positive PCR amplicons were characterized by sequencing and comparison in a phylogenetic analysis to an independent sample of RABV sequence data from insectivorous bats across the US. Parameters for the model of sequence evolution

were estimated using MODELTEST (Posada and Crandall, 1998), and AIC values indicated that the TrN+I model provided the best fit to the data (AIC=1944.4, K=6, -lnL=966.2). Markov Chain Monte Carlo (MCMC) simulations were run in a Bayesian framework for 2.4 million generations, sampling every 1,000 generations and using a burn-in period of 200,000 generations using MR. BAYES V.3.1 (Ronquist and Huelsenbeck, 2003). Posterior probabilities represent the frequency (percentage out of 2002 trees) of phylogenetic group associations for nodes of interest.

Statistical Analyses

Rabies VNA seroprevalence was treated as a binomial response variable for all analyses. For statistical analyses, the seroprevalence data were partitioned into two cohorts: adult bats (N=316) and juvenile bats (N=45). A series of hierarchical logistic models were tested using SAS v.9.1 (SAS Institute, Inc., Cary, NC) to investigate significant ecological predictors of rabies VNA seroprevalence. The central question focused on testing for effects of roost type and seasonal variation on rabies VNA seroprevalence. A nested mixed logistic model (PROC GLIMMIX) was used to control for variation among sites that represent the two roost types (bridge vs. bat house) sampled in this study, with site treated as a random effect nested within roost type, and fixed effects of roost type, period of sampling, gender, and reproductive condition (a=0.05). In the absence of significant effects of roost type, models were simplified to non-nested mixed logistic models, with site still treated as a random effect (α=0.05). In the nested and non-nested models, individual body condition was tested as a covariate.

All two and three-way fixed effect and covariate interactions were tested (α=0.05). Non-significant covariate-fixed effect interactions were removed from the model prior to testing fixed effects (Engqvist, 2005). Marginally significant ($0.05<\alpha<0.10$) fixed effect interactions were retained in the models. For models with significant fixed effects, pair-wise contrasts among all levels of the fixed effect were tested (α=0.05). Figures 2.4 and 2.5 show proportions of rabies VNA seroprevalence, with the upper 95% confidence interval displayed above each proportion.

Although tested as a covariate in the seroprevalence models, body condition data were also analyzed separately in adult cohorts of male and female bats, and separately for the juvenile cohort. A nested mixed ANOVA model was used to test for roost type effects on body condition among adult female and male bats at bridge and bat house colonies. All nested, fixed, and random effects were treated as described above. As time of capture was occasionally later for bat house colonies compared to bridge colonies, we expect that body condition may be greater in bats captured from bat-house colonies due to potential foraging prior to capture. A nested mixed ANOVA model was used to test for roost type effects on body condition of the juvenile cohort (N=48) across all colonies during the Late period. In the absence of significant roost type effects, nested mixed ANOVA models were simplified to non-nested form, and data were re-analyzed. Tukey's post-hoc means separation test was used to compare all pair-wise levels for significant fixed effects (α=0.05).

Model testing was also conducted with the adult and juvenile body condition and rabies VNA seroprevalence data sets. Akaike Information Criterion (AIC) values were

used to rank all possible models (Burnham and Anderson, 2002), and determine the optimal set of predictors to explain the data.

Results

Colony sizes were highly variable (est. 200-20,000), and appear to positively correlate with the physical size of roosts. Southeastern myotis bats were commonly found inhabiting roosts with *T. brasiliensis* throughout colonies in Florida, and big brown bats (*Eptesicus fuscus*) were also consistently found sharing bridge roosts with *T. brasiliensis* and *M. austroriparius* in northern FL (BCB, SACB, TCB; Figure 2.1). Evening bats (*Nycticeus humeralis*) lived in a bat house that was in proximity to the free-tailed bat house colony sampled in Quitman, GA (QBH), although this species was infrequently captured during mist-net sampling at this location. In Florida, different species within a shared roost typically formed spatially exclusive aggregations, but among northern bridge colonies, the capture of all species (*T. brasiliensis*, *M. austroriparius*, and *E. fuscus*) in mist-nets during the evening emergence was common across the sampling season. Compared to the one to two hour-long evening emergences from colonies in Texas, emergence events in the southeast were typically less than 30 minutes in duration, although our physical presence underneath bridge colonies may have altered emergence behavior for some individuals. Similar proportions of adult male and female bats were captured from most bridge and bat-house colonies, although the largest bridge colony (AUB) was highly skewed (27:1) towards females. Among northern Florida bridge colonies, two bats were re-captured in June (SACB, BCB), one bat was re-captured in July (TCB), and two bats were re-

captured in August (TCB, BCB). All re-capture events occurred at the original colony of sampling, and all re-captures occurred in bridge colonies that had small estimated colony sizes (i.e. 200-500) (Gore and Studenroth, 2005).

A total of 425 Brazilian free-tailed bats were sampled for rabies VNA between May and August 2006. Rabies VNA titers were determined from 373 samples, and 98% of values were between 0-2 IU/ml (Table 2.1, Table 2.2). None of the bats presented clinical signs of RABV infection during handling, sampling, or release. From RT-PCR testing of 295 swab samples, the saliva of one bat (0.33%) tested positive for RABV RNA (bat #3450, non-reproductive adult female, BCB, August 12). Sequence data from the PCR amplicon suggests close affinity with other *T. brasiliensis* RABV isolates from the US (Figure 2.3).

VNA Seroprevalence – Adults

A total of 316 adult bats were tested for rabies VNA at bridge (N=204) and bat-house roosts (N=112). Rabies VNA seroprevalence among adult bats from the southeastern US during 2006 (38% [33.1-43.8]) was not significantly different from estimates for adults from Texas during 2005 (40% [35.9-44.6]) (Turmelle et al., *In Press*). Within the southeastern US, rabies VNA seroprevalence was significantly higher among bats roosting in bat-houses (51% [41.8-60.0]) compared to bridges (31% [25.4-38.0]) (**df=1, 4, F=13.28, p=0.02**) (Figure 2.4).

In the nested mixed logistic model comparing adult bats (no. sites=6, n=316; Table 2.3), a significant roost type by period interaction was detected (**df=2, 302, F=4.47, p=0.01**; site [roost]=0.009 [±0.08]), and gender was also significantly predictive

of VNA seroprevalence (**df=1, 302, F=4.08, p=0.04**) (Figure 2.4). Data were subset by roost type for subsequent analyses.

In the non-nested mixed logistic model comparing adult bats living in bridge colonies (no. sites=4, n=204; Table 2.4), period of sampling was a significant predictor of VNA seroprevalence (**df=2, 196, F=5.03, p=0.007**; site=0.01 [±0.09]), and log-transformed body condition of individual bats was also a marginal covariate (**df=1, 196, F=2.79, p=0.10**). In a subset of female bats (n=124), reproductive status was a significant predictor of VNA seroprevalence (**df=2,118, F=3.71, p=0.03**; site 0.04 [±0.20]), and contrasts indicate that pregnant (p=0.02) and lactating (p=0.03) female bats are more likely to be VNA seropositive compared to non-reproductive females. However, period of sampling was not significant in the model for the subset of male bats (n=77; Table 2.4) (**df=2, 70, F=0.71, p=0.49**; site=0.15 [±0.36]).

In the non-nested logistic model comparing adult bats living in bat house colonies (no. sites=2, n=112; Table 2.5), period of sampling was not a significant predictor of VNA seroprevalence (**df=2, 105, F=2.16, p=0.12**; site=1.6e-19), but gender was a marginal predictor (**df=1, 105, F=3.26, p=0.07**). Contrasts indicate that male bats were more likely to be seropositive compared to female bats. In a subset of female bats (n=63), reproductive status was not a significant predictor of with VNA seroprevalence (**df=2, 59, F=0.49, p=0.62**; site=3.56e-22). In a subset of male bats (n=46), period of sampling was not a significant predictor of VNA seroprevalence (**df=2, 41, F=2.04, p=0.14**; site=4e-20).

VNA Seroprevalence – Juveniles

In the nested mixed logistic model comparing juvenile bats from four bridge and two bat house colonies during the Late period (n=45; Table 2.6), roost type was not a significant predictor of VNA seroprevalence (**df=1,4, F=0.10, p=0.76**; site [roost]= 1.59e-19) (Figure 2.5), but body condition was a marginal covariate (**df=1, 38, F=2.92, p=0.10**). In the non-nested logistic model, gender was not a significant predictor of VNA seroprevalence (**df=1, 37, F=0.61, p=0.44**), but body condition was a marginal covariate (**df=1, 37, F=3.42, p=0.07**; site=2.69e-18), and heavier juvenile bats were more likely to be seropositive.

VNA seroprevalence among juvenile and adult cohorts was compared during the Late period using a nested mixed logistic model (n=98), but no significant age effect was observed (**df=1, 90, F=0.38, p=0.54**; site [roost]=1.4e-18) (Table 2.7).

Body Condition – Adults

The nested mixed ANOVA model explained 39% of the variation in body condition among adult bats (no. sites=6, n=362; Table 2.8). The residuals of three bats were outliers (2 females, 1 male) in the ANOVA model, and these bats were excluded post-hoc to achieve normality of residuals to the model. The three-way interaction between roost type, period, and gender was significant (**df=2, 350, F=6.44, p=0.002**; site [roost]=0). Data were subset by gender for subsequent analyses, as female body condition is likely to be highly sensitive to changing reproductive status, whereas males are uniformly non-reproductive during the sampling season.

The nested mixed ANOVA model explained 22% of the variation in body condition among adult female bats (no. sites=6, n=213; Table 2.9). There was a significant interaction between roost type and reproductive status (**df=2, 201, F=4.87, p=0.009**; site [roost] z=0.89, p=0.19). The body condition of pregnant females in bat houses was significantly higher than pregnant females from bridge colonies, but there was no significant effect of roost type on the body condition of lactating or non-reproductive (post-lactating) female bats. Controlling for differences between roosts, body condition was highest for pregnant bats, dropped significantly during lactation, and was slightly higher in non-reproductive females compared to lactating females (Figure 2.6).

A nested mixed ANOVA model explained 52% of the variation in body condition among adult male bats (no. sites=5, n=143; Table 2.9). Period of sampling was the only significant predictor in the model (**df=2, 136, F=67.63, p<0.0001**; site [roost] z=0.75, p=0.23), and contrasts indicate that body condition of males is significantly lower in during the Early period (May) compared to Mid (June, July) and Late (August) periods (Figure 2.7).

Body Condition – Juveniles

The nested mixed ANOVA model explained 48% of the variation in body condition among juvenile bats during the Late period (no. sites=6, n=48; Table 2.10). Roost type was not a significant predictor in the model (**df=1, 4.6, F=2.23, p=0.20**; site [roost] z=1.15, p=0.12) (Figure 2.8). A non-nested mixed ANOVA model with only gender as a fixed effect, and site as a random effect, explained the same percentage of

variation as the nested model, and gender was a marginal predictor of juvenile body condition (**df=1, 41, F=2.80, p=0.10**; site z=1.30, p=0.10). Male juvenile bats were slightly heavier than females during the Late period.

The forearm length (i.e. body size) and body condition indices among juvenile and adult cohorts were tested during the Late period using a nested mixed ANOVA model (n=104). Significant age effects were detected for body condition among adult and juvenile bats (**df=1, 98.2, F=75.96, p<0.0001**; site [roost] z=1.22, p=0.11), but not in body size among the same cohorts (**df=1, 98.5, F=0.35, p=0.56**; site [roost] z=0.42, p=0.34) (Table 2.11). Despite similar body size, juvenile bats were much lighter compared to adult bats during the Late period.

Comparisons of model AIC values for VNA seroprevalence and body condition data sets converged on identical sets of significant predictors when compared to the statistical tests for partitioned data (Table 2.12 – Table 2.15). The best model of VNA seroprevalence among adult bats did not include body condition as a covariate, but the best models of VNA seroprevalence among juvenile bats did (Table 2.12, Table 2.13).

Discussion

Population Ecology

Several Brazilian free-tailed bats were re-captured during the Mid and Late periods from the original bridge colonies where marked during the 2006 field season. Additionally, three bats were recaptured at two of the northern Florida bridge roosts in May 2007, all with tattoos from the original colony where marked in 2006 (SACB [n=1], BCB [n=2]). However, all re-capture events during 2006 and 2007 from the

southeastern US were only observed among three small bridge colonies (Gore and Studenroth, 2005). For larger colonies in Florida or Texas (i.e. greater than 10,000 bats), our capture and marking sample sizes were likely inadequate to successfully recapture marked bats on successive visits, as suggested from a study in Texas where 0.8% (177 of 21,140) of marked bats were recaptured within a season (Davis et al., 1962). These data suggest site fidelity among the roosts sampled both within and across years, but many more individuals would need to be marked and recaptured to robustly assess the degree of within season and across season fidelity of bats to different roosts.

This study is the first to systematically investigate ecological variation, and RABV exposure, across several colonies of Brazilian free-tailed bats in the southeastern US. Despite colony sizes that are orders of magnitude lower compared to colonies from Texas, and the potential absence of a long-distance autumn migration, this study corroborates previous suggestions (Sherman, 1937; Wilkins, 1989) that female Brazilian free-tailed bats in the southeastern US have similar reproductive life history compared to Texas colonies during concurrently timed sampling across years. Brazilian free-tailed bats in the southeastern US also may have greater interspecific interactions with other bats compared to the colonies sampled in Texas, although colonies of Brazilian free-tailed bats in the northwestern US also have substantial interspecific interactions (Krutzsch, 1955). Despite potential for interspecific competition, the smaller colonies of Brazilian free-tailed bats in the southeastern US may experience lower intraspecific competition compared to the larger colonies in Texas and NM (Betke et al., 2008; Constantine, 1967a). Rabies surveillance was not conducted among other bat species

commonly captured in this study, including *E. fuscus* and *M. austroriparius*, which precluded testing the effects of roost cohabitation on RABV epizootiology in Brazilian free-tailed bats or other species. Within the bridge roosts of northern Florida, colonies of *M. austroriparius* appeared to be of similar size (~200-500 bats) compared to Brazilian free-tailed bats, but *E. fuscus* colonies in the same bridges were about one-quarter the size of Brazilian free-tailed or *M. austroriparius* colonies (~50 bats) (Gore and Studenroth, 2005). A barn roost was visited twice in Thomasville, GA, in June and September 2007. Free-tailed bats and big brown bats were observed roosting in the barn on both occasions, but groups of both species were not spatially exclusive (personal observations). The observation of pups for both species confirms the use of this barn as a maternity colony, although relative proportions of individuals for both species were consistent with other locations sampled in Florida, with greater numbers of Brazilian free-tailed bats. However, other bat species were not observed in a large (~1000) residential colony of Brazilian free-tailed bats in Forsyth, GA, visited in March 2006 and a small (~100) bat house colony in Deltona, FL, visited in May of 2006. A colony (~500 bats) living in the belfry of a residential housing building in downtown Little Rock, AR, was visited monthly from May-August 2006, and also contained only Brazilian free-tailed bats. Although Brazilian free-tailed bats occasionally share roosts with cave myotis bats (*Myotis velifer*) and more rarely with ghost-faced bats (*Mormoops megalophylla*) in Texas caves (Davis et al., 1962; Jameson, 1959), the number of Brazilian free-tailed bats in these colonies appears to grossly outnumber abundance of either of the other two species by several orders of magnitude, likely leading to negligible interspecific interaction from the perspective of the free-tailed bats. However,

in California, it has been noted that Brazilian free-tailed bats are often found roosting with other species, including *E. fuscus*, pallid bats (*Antrozous pallidus*), and several western *Myotis* species (Krutzsch, 1955). Thus, in the southeastern and northwestern parts of their geographic range, it may be more common to find Brazilian free-tailed bats roosting with other species. The degree of spatial overlap within colonies, and relative colony sizes of the different species involved, are expected to impact contact networks among and between species, and may impact RABV epizootiology.

An independent study found that Brazilian free-tailed bats and big brown bats rarely exhibit evidence of cross-species transmission of RABV to other bat species (Streicker et al., *In Prep.*). However, other studies have found that infection prevalence may be amplified when multiple reservoir species interact, as has been noted among carnivore reservoirs of RABV (Guerra et al., 2003), and carnivore reservoirs of canine distemper virus (Craft et al., 2008). Recent observations in Arizona have demonstrated cross-species transmission events of big brown bat RABV to skunks (Leslie et al., 2006), and cross species transmission of RABV has been noted among bat and terrestrial carnivore species in Colorado (Shankar et al., 2005). The oropharyngeal swab data from this study does not suggest cross-species transmission of RABV within the mixed species colonies, but concurrent surveillance for all species within roosts should be a priority in future studies.

Adults

Significant roost type effects were observed among bridge and bat house colonies in Florida and Georgia. As both large (~10,000) and small colonies (~200-500)

were sampled among bat house and bridge roosts, the differences in patterns of VNA seroprevalence are unlikely to be driven by colony size differences between roosts, in contrast to observations from the Texas study (Chapter 1). It is unlikely that minor differences in the time of capture between bridge and bat house colonies would have resulted in the capture of significantly different cohorts of individuals with respect to VNA seroprevalence given the small size of most colonies. Bat house colonies may have higher RABV infection pressure, or perhaps higher immunocompetence for clearing peripheral infection (i.e. greater seroconversion despite similar infection pressure). Similar to the Texas study, contact rates among bridge colonies of bats may be lower compared to bat house colonies, due to the spatial configuration of animals within the roost. Although bat houses do not have a radial configuration that is observed in cave colonies, the stacking of several linear slats side by side within the bat house may lead to greater contact during emergence from and return into the roost.

Across all colonies in Florida and Georgia, males were significantly more likely to be seropositive, but this pattern was driven by primarily by gender differences among bat house colonies. Bat house colonies had higher and more uniform levels of VNA seroprevalence across the reproductive season compared to bridge colonies. In bat house colonies, males were more likely to be seropositive, but VNA seroprevalence did not vary seasonally among adult female or male cohorts. Contrastingly, while gender was not a predictor of VNA seroprevalence among bridge colonies of bats, significant seasonality was detected and resulted from heightened VNA seroprevalence among pregnant and lactating females. A study of Lagos Bat Virus (LBV) VNA seroprevalence, among bats across several sites in Kenya, reported significantly higher numbers of

seropositive males compared to females for Egyptian fruit bats (*Rousettus aegyptiacus*), but only marginally higher numbers of seropositive males among straw-colored fruit bats (*Eidolon helvum*) (Kuzmin et al., 2008). As gender differences in VNA seroprevalence among Brazilian free-tailed bats were not apparent from a recent study in Texas (Turmelle et al., *In Press*), and a previous study at Carlsbad Cavern, NM (Constantine et al., 1968), the existing data are inconclusive as to whether male bats are more susceptible to lyssavirus infection. Nor are there controlled studies that address this question through experimental infection. In summary, the interactions between roost type, life history, and gender are important predictors of rabies VNA seroprevalence among adult Brazilian free-tailed bats in bridge and bat house roosts in Florida and Georgia, but predictors are not consistent across different types of roosts.

Seasonal effects on body condition were significant among adult bats across colonies in Georgia and Florida. Changes in the body condition of adult female bats were consistent with expected changes in energetic demands and gestation, where pregnant female bats were heaviest, and lactating and non-reproductive females were lighter (Kunz et al., 1995). However, during pregnancy and non-reproductive phases, females captured from bat house colonies appeared to have higher body condition compared to females captured from bridge roosts during the same periods. This pattern may be biased by the difference in capture times between bridge and bat house roosts, where females from bat house colonies may have had two to three hours of foraging activity prior to capture. Despite the differences observed among adult females from different roost types and expected bias due to capture time, roost-type effects on body condition were not observed among adult male bats from bridge and bat house

colonies. Alternatively, roost type effects may exist for male cohorts, but could be obscured by inflated values due to later sampling at bat house roosts. Seasonal variation was detected in male body condition, with lower values during the Early period, when most females are pregnant (Figure 2.2). Interestingly, this pattern was also observed in males from the single cave colony, but not among three bridge colonies, sampled in Texas (Figure 1.6). The energetic demands of adult female Brazilian free-tailed bats are greatest during pregnancy and lactation (Kunz et al., 1995), and may lead to strong competition between the sexes when resources are limited. Data on insect densities are scarce for the southeastern US (compared to Texas), but low prey availability may contribute compromised adult male body condition during female pregnancy (i.e. May), but not during female lactation (i.e. June, July) when prey may be more abundant in both regions.

Juveniles

Roost-type effects were not observed for VNA seroprevalence data among juvenile bats sampled during the Late period (i.e. August), and seroprevalence estimates among juvenile bats were not significantly different from the adult cohort during this period. As was observed in the Texas study (Turmelle et al., *In Press*), RABV exposure for the volant juvenile cohort appears identical to the adult cohort, which is contrary to the results of a study with this species from Lava Cave, NM, during August (Steece and Altenbach, 1989), but similar to results during August from Carlsbad Cavern, NM (Constantine et al., 1968). The differences in patterns between

the studies presumably reflect differences in sampling technique (e.g. capture in roost versus during emergence).

There was a slight association of VNA seroprevalence with body condition, and heavier bats were more likely to be seropositive in nested and non-nested logistic models. This pattern contrasts observations from juvenile bats in Texas, where lighter juveniles were more likely to be seropositive when controlling for sampling period. All of the juveniles captured during the Late period (i.e. August) in the southeastern US were at least 6 weeks of age (given synchronized parturition within the first two weeks of June), but were significantly lighter than adult bats during this period despite having similar body size. Despite differences in the time of capture between roosts, no significant roost-type effects were detected for the body condition of juvenile bats captured from bat house or bridge colonies. In comparisons across all juvenile bats, male juveniles were slightly heavier than female juveniles, but it is unclear whether this trend is biologically significant given the limited sampling across gender for this cohort. These data indirectly suggest relatively similar growth rates of pups among colonies in Florida, Georgia, and Texas. A study from a cave in Texas (JRC in Chapter 1) determined that a logistic model provided the best fit to the growth rates of young, and that adult size (i.e. forearm length) is typically reached by the sixth week post-partum (Kunz and Robson, 1995). Longitudinal recapture of pups during the first six weeks post-partum would be needed to address variation in the growth trajectories of pups in different regions and roost types.

Conclusions

Colonies of Brazilian free-tailed bats in the southeastern US were much smaller in size, and were much more likely to cohabit with other bat species in roosts compared to colonies in Texas. Similar to the Texas study, bats with rabies VNA were observed at all of the colonies sampled and across all age and gender cohorts. Roost type, gender, and seasonal life history were important predictors of VNA seroprevalence and body condition among Brazilian free-tailed bat colonies in Florida and Georgia. However, additional sampling across years is needed to test whether currently identified trends are robust predictors of VNA seroprevalence and RABV infection pressure. Although several studies have conducted longitudinal studies of lyssavirus infection dynamics across years, scarce data on season, age, and gender variation in VNA seroprevalence are typically provided in these studies (Amengual et al., 2007; Serra-Cobo et al., 2002; Vazquez et al., 2006). Evidence from several studies of rabies VNA seroprevalence in Brazilian free-tailed bats (Constantine et al., 1968; Steece and Altenbach, 1989; Turmelle et al., *In Press*) demonstrates that VNA seroprevalence can vary seasonally by age, gender, and life history of bats. Presumably, annual fluctuations reported among other lyssavirus studies with insectivorous bats in Europe reflect annual fluctuations in seroprevalence, rather than artifacts of variation in time of sampling across years. Future studies of bat epizootiology in wild populations should consider both within season and across season time scales when conducting longitudinal surveillance.

Chapter 3

Local and landscape factors contributing to rabies virus exposure and body condition in Brazilian free-tailed bats in the southern US

Introduction

Brazilian free-tailed bats (*Tadarida brasiliensis*) are widely distributed in the New World, and occur in the southern United States (US), all of Central America, along the coastal provinces of northern South America, and throughout most of southern South America (Wilkins, 1989). Many Brazilian free-tailed bats are migratory, with some populations engaging in long distance seasonal migrations of up 1280 km (Cockrum, 1969; McCracken et al., 1994). In extreme eastern and western parts of the geographic range in the US, however, many of these bats do not engage in long-distance migration, but remain in regional colonies year-round (Krutzsch, 1955; Lee and Marsh, 1978; Sherman, 1937). Brazilian free-tailed bats occupy a variety of man-made and natural roost structures in the southern US. In caves of the southwestern US, this species aggregates in maternity colonies of up to 10s of millions of bats, and colonies of up to a million bats are increasingly common under highway bridges in the southwestern US (Betke et al., 2008; Keeley and Keeley, 2004; McCracken, 2003). Across the southern US, colonies of 100s to 1000s are often found in buildings, bridges, culverts, and bat houses (Constantine, 1967a; Davis et al., 1962; Gore and Studenroth, 2005; Krutzsch, 1955; Scales and Wilkins, 2007; Sherman, 1937). Colonies of Brazilian free-tailed bats from Louisiana and eastward in the southeastern US have previously been classified as

a separate subspecies (*T. b. cynocephala*), due to behavioral and morphological differences, and molecular variation at one allozyme locus (Carter, 1962; Owen et al., 1990). However, a comprehensive study of mitochondrial DNA variation across several colonies from the US and Mexico suggests a lack of regional haplotypic spatial structure, and lends no support for specific or sub-specific boundaries within *T. brasiliensis* in North America (Russell et al., 2005).

Brazilian free-tailed bats have been linked to 22% (8 of 36) of bat-associated cases of human rabies across the US since 1980, with cases occurring across their geographic range (i.e. Alabama [1], Arkansas [1], California [3], Georgia [1], and Texas [2]) (Blanton et al., 2008; Messenger et al., 2002). RABV-infected Brazilian free-tailed bats have been detected from natural colonies across their geographic range in the southern US (Burns et al., 1956a; Burns and Farinacci, 1955; Burns et al., 1956b; Dean et al., 1960; Glass, 1959; Maddy et al., 1958; Richardson et al., 1966; Schneider et al., 1957), although systematic surveillance of apparently healthy adult Brazilian free-tailed bats from maternity colonies in Texas and New Mexico (NM) has documented low prevalence of RABV infection (< 1%) (Constantine et al., 1968; Steece and Altenbach, 1989). Although natural colonies of bats in the US are presumed to have similar prevalence of RABV infection across species, Brazilian free-tailed bats appear to have some of the highest levels of virus neutralizing antibody (VNA) seroprevalence compared to other bat species in the US (Constantine et al., 1968; Steece and Altenbach, 1989; Trimarchi and Debbie, 1977; Turmelle et al., *In Press*).

There have been many recent studies on geographic, seasonal, and annual variation in lyssavirus epizootiology within and across bat species. Longitudinal studies

on European Bat Lyssavirus 1 (EBLV-1) epizootiology have documented annual and geographic trends among multiple mixed and single species colonies of vespertilionid bats in Spain (Amengual et al., 2007, 2008; Perez-Jorda et al., 1995; Serra-Cobo et al., 2002; Vazquez et al., 2006; Vazquez-Moron et al., 2008). Studies addressing geographic trends of VNA seroprevalence in Lagos Bat Virus (LBV) have been conducted with insectivorous and fruit bat species across several locations in Kenya (Kuzmin et al., 2008) and Ghana (Hayman et al., 2008). Additionally, European Bat Lyssavirus 2 (EBLV-2) surveillance has been conducted among several colonies of Daubenton's bats (*Myotis daubentonii*) in Scotland (Brookes et al., 2005). Despite intensive longitudinal studies of RABV infection in Brazilian free-tailed bats at two caves in NM (Constantine et al., 1968; Steece and Altenbach, 1989), and one broader geographic study from 1955 (Burns et al., 1956a), there have been no published studies that systematically address both spatial and temporal variation in RABV exposure among North American bats.

Brazilian free-tailed bats are an important reservoir of RABV in North America, have extensive human contact that has resulted in cases of indigenous human RABV infection in the US, and demonstrate extensive roosting, behavioral, and ecological variation across their geographic range. This combination of factors provides a unique system for investigating spatiotemporal predictors of disease prevalence. This study compares local and regional factors affecting RABV exposure and body condition among Brazilian free-tailed bat colonies that were sampled from caves, bridges, and bat house roosts in several locations across seasonal life history periods, from south-central Texas during 2005, and Florida and Georgia during 2006.

Methods

Animal Sampling

This study combines data that are presented in Chapters 1 and 2, across 12 natural colonies of Brazilian free-tailed bats in the southern US (Figure 3.1). Sampling was conducted from May through August in 2005 for the colonies in Texas, with additional opportunistic sampling during September and October 2005, September 2006, and July 2008, for subsets of colonies (FC, SCB, DBC, MB). Systematic sampling was conducted from May through August in 2006 for the colonies in Florida and Georgia, with opportunistic sampling during May 2007 for a subset of colonies (BCB, SACB). Comparisons presented in this study include only the sampling that occurred from May-August in 2005 for Texas colonies, and May-August in 2006 for Florida and Georgia colonies. However, reference is made to sampling done in other months and years to lend evidence to the stability of estimates and trends across years. The data suggest that seasonal life history schedules of adult females are similar between these two regions (Figure 3.2).

Statistical Analyses

Rabies VNA seroprevalence was treated as a binomial response variable for all analyses. For statistical analyses, the seroprevalence data were partitioned into two cohorts: adult bats (N=779) and juvenile bats (N=71).

A series of hierarchical logistic models were tested using SAS v.9.1 (SAS Institute, Inc., Cary, NC) to investigate significant ecological predictors of rabies VNA seroprevalence. The central question focused on testing for effects of roost type and region on rabies VNA seroprevalence, particularly among adult bats. A nested mixed logistic model (PROC GLIMMIX) was used to control for variation among sites that represent the three roost types (cave, bridge, bat house) and the two regions (Texas, Florida/Georgia) sampled, with site treated as a random effect nested within roost type or region, and fixed effects of roost type, region, period of sampling, gender, and reproductive status (α=0.05). In the absence of significant effects of roost type or region, models were simplified to non-nested mixed logistic models, with site still treated as a random effect (α=0.05). In the nested and non-nested models, individual body condition was tested as a covariate. All two and three-way fixed effect and covariate interactions were tested (α=0.05). Non-significant covariate-fixed effect interactions were removed from the model prior to testing fixed effects (Engqvist, 2005). Marginally significant ($0.05<\alpha<0.10$) fixed effect interactions were retained in the models. For models with significant fixed effects, pair-wise contrasts among all levels of the fixed effect were tested (α=0.05).

Although tested as a covariate in the seroprevalence models, body condition data were also analyzed separately in male and female adult cohorts, and for the juvenile cohort, to investigate roost type, region, gender, and seasonal effects on body condition. A nested mixed ANOVA model was used to test for roost type differences in body condition among adult and juvenile bats from three different roost types (cave, bridge, bat house) and two different regions (Texas, Florida/Georgia). All nested, fixed,

and random effects were treated as described above. In the absence of significant roost type effects, nested mixed ANOVA models were simplified to non-nested form, and data were re-analyzed. Tukey's post-hoc means separation test was used to compare all pair-wise levels for significant fixed effects (α=0.05).

Model testing was also conducted with the adult and juvenile body condition and rabies VNA seroprevalence data sets. Akaike Information Criterion (AIC) values were used to rank all possible models (Burnham and Anderson, 2002), and determine the optimal set of predictors to explain the data.

Results

VNA Seroprevalence – Adults

In the nested mixed logistic model of VNA seroprevalence comparing adult bats across the southern US (no. sites=9, n=779; Table 1.1, Table 2.1, Table 3.1), a significant roost type by period interaction was detected (**df=2, 756, F=3.31, p=0.01**; site [roost]=0.26 [±0.16]). Contrasts indicate significant differences between VNA seroprevalence at bridge and bat house colonies (p=0.03), and between cave and bat house colonies (p=0.01), during the Early period (Figure 3.3). Contrasts between roosts during other time periods were non-significant.

In the nested mixed logistic model of VNA seroprevalence among adult bats from bridge colonies only (n=435; Table 3.1), a significant region by period interaction was detected (**df=2, 420, F=3.30, p=0.04**; site [region]=0.09 [±0.10]). Contrasts indicate that VNA seroprevalence among adult bats from Texas bridge colonies is significantly higher

compared to bridge colonies in Florida during the Late period (p=0.001) (Figure 3.4), although VNA seroprevalence was generally higher among Texas colonies across all time periods.

VNA Seroprevalence – Juveniles

The nested mixed logistic model of VNA seroprevalence among juvenile bats during the Late period was tested (no. sites=11, n=71; Table 3.2), but did not converge. In a reduced model comparing only bridge and bat house colonies (no. sites=8; n=64), roost type was not a significant predictor of juvenile VNA seroprevalence during the Late period (**df=1, 6, F=0.05, p=0.83**; site [roost]=0.33 [±0.56]) (Table 3.2, Figure 3.5). In a non-nested model including all juveniles (n=71), gender was not a significant predictor of VNA seroprevalence during the Late period (**df=1, 59, F=2.50, p=0.12**; site=0.24 [±0.43]). Body condition was initially tested in the non-nested model as a covariate, but was non-significant and removed (p=1.0).

In the nested mixed logistic model of VNA seroprevalence among juvenile bats living in bridge colonies during the Late period (no. sites=6, n=54; Table 3.2), marginally significant regional effects were detected (**df=1, 4, F=6.40, p=0.06**; site [region]=8.67e-19). Juvenile bats from bridge colonies in Texas had higher VNA seroprevalence compared to bridge colonies in Florida during the Late period (Figure 3.6**).**

Body Condition – Adults

A nested mixed ANOVA model explained 41% of the variation in body condition among adult female bats across the southern US (n=545; Table 3.3). A significant roost type by reproductive status interaction was detected (**df=4, 532, F=5.30, p=0.0003**; site [roost] z=1.43, p=0.08). Contrasts indicate that, during the lactation and non-reproductive phases, the body condition of female bats from cave colonies was significantly lower compared to female bats from bridge or bat house colonies (Figure 3.7).

The nested mixed ANOVA model explained 20% of the variation in body condition among adult female bats from bridge colonies (n=258; Table 3.3). The interaction between reproductive status and region was significant (**df=2, 224, F=3.23, p=0.04**; site [region] z=0.91, p=0.18). However, contrasts do not suggest significant regional effects on body condition during pregnancy, lactation, or non-reproductive periods. Body condition of adult female bats from bridge colonies is highest during pregnancy, lower during lactation, and is lowest during the non-reproductive phase (Figure 3.8).

A nested mixed ANOVA model explained 23% of the variation in body condition among adult male bats in the southern US across time periods (n=301; Table 3.4). Roost type was not a significant predictor in the model (**df=2, 17.2, F=1.02, p=0.38**), but there was a marginal interaction between roost type and period (**df=4, 290, F=2.17, p=0.07**). Period of sampling was the most significant predictor of male body condition (**df=2, 289, F=21.01, p<0.0001**; site [roost] z=0.73, p=0.23). Contrasts indicate that

body condition of adult male bats is lowest during the Early period, and is uniformly higher during the Mid and Late periods (Figure 3.9).

As roost type does not appear to significantly impact male body condition, two nested mixed ANOVA models were tested for regional comparisons, one including all colonies of male bats (no. sites=9, n=301), and a different model including male bats across bridge colonies only (no. sites=6, n=229; Table 3.4). In both models, the region by period interaction was highly significant (**n=301, df=2, 294, F=21.66, p<0.0001**; site [region]=1.04, p=0.15) (**n=229, df=2, 222, F=21.49, p<0.0001**; site [region] z=0.96, p=0.17). In both models, contrasts indicate that male body condition was significantly lower during the Early period in Georgia and Florida colonies, compared to all other time periods and the Texas colonies. However, during the Mid and Late periods, male body condition was higher in the Georgia and Florida colonies compared to Texas colonies (Figure 3.10). In the model including males sampled from cave, bridge, and bat house colonies, body condition significantly varied by period. However, in comparisons among bats from bridge colonies only, the body condition of males was variable across periods in the Georgia and Florida colonies, but uniform across periods in Texas.

Body Condition – Juveniles

A nested mixed ANOVA model explained 61% of the variation in body condition among juvenile bats in the southern US during the Late period (no. sites=12, n=84, Table 3.5). Roost type was not a significant predictor of juvenile body condition (**df=2, 9, F=2.79, p=0.11**; site [roost] z=1.79, p=0.04) (Figure 3.11). In the non-nested model, gender was a significant predictor of juvenile body condition during the Late period

(**df=1, 71, F=4.20, p=0.04**; site z=2.03, p=0.02). Male juvenile bats were significantly heavier than female juvenile bats.

A nested mixed ANOVA model was tested for regional effects on body condition of juvenile bats across all colonies in the southern US (no. sites=12, n=84), and in a separate model with juvenile bats across bridge colonies only (no. sites=7, n=62; Table 3.5). In both models, region was a significant predictor of juvenile body condition during the Late period (**df=1, 10, F=12.01, p=0.006**; site [region] z=1.63, p=0.05) (**df=1, 5, F=7.56, p=0.04**; site [region] z=1.25, p=0.11). In either model, juvenile bats from Florida and Georgia colonies were heavier than juveniles from Texas colonies (Figure 3.12). Among all juveniles (n=84), region was a significant predictor of juvenile body condition during the Late period (**df=1, 9.86, F=10.81, p=0.008**; site [region] z=1.67, p=0.05), and gender was marginally associated (**df=1, 74.2, F=3.81, p=0.06**).

Comparisons of model AIC values for VNA seroprevalence and body condition data sets converged on identical sets of significant predictors when compared to the statistical tests for partitioned data (Table 3.6 – Table 3.13). The best regional and roost type models of VNA seroprevalence among adult or juvenile bats did not include body condition as a covariate (Table 3.6, Table 3.7, Table 3.8, Table 3.9)

Discussion

Landscape Spatial and Temporal Trends

Data from this study suggest that RABV exposure, as evidenced by VNA seroprevalence, is highly variable across space and time at local scales, despite being a spatially and temporally stable pressure on Brazilian free-tailed bat populations at

landscape scales. Observations of widespread geographic RABV infection are consistent with the population genetic structure of this host. Brazilian free-tailed bats are capable of rapid and long-distance dispersal, in addition to exhibiting large effective population sizes in many regions, both of which contribute to a lack of spatial genetic structure across their geographic range in North America (McCracken and Gassel, 1997; Russell et al., 2005). High gene flow and dispersal capabilities suggest strong connectivity between regional populations, perhaps leading to patterns of RABV epizootiology that are synchronous across the landscape (Hampson et al., 2007). Temporal stability of RABV epizootiology in Brazilian free-tailed bats has been demonstrated through multiple studies over the past 50 years (Burns et al., 1956a; Constantine et al., 1968; Steece and Altenbach, 1989; Turmelle et al., *In Press*), and appears consistent with epizootiological stability of EBLV-1 infection across years in greater mouse-eared bats (*Myotis myotis*) from Spain (Amengual et al., 2007; Serra-Cobo et al., 2002). Unfortunately, most studies have either focused on intra- or inter-annual epizootiology, although current evidence suggests evidence of significant intra-annual but minimal inter-annual fluctuations in RABV epizootiology in Brazilian free-tailed bats.

Impact of Migration

Long-distance migration is a costly activity for bats, but temporally abundant resources may motivate many species to undertake seasonal migration (Fleming and Eby, 2003). In certain parts of their geographic range, female Brazilian free-tailed bats

annually migrate from Mexico to the United States for concurrently timed parturition and ephemerally high insect abundance. Many tropical bat species undergo short and long-distance migrations to track spatial and seasonal variation in fruit availability, as occurs with the straw-colored fruit bat (*Eidolon helvum*) (Richter and Cumming, 2006) across sub-Saharan Africa, and with the large flying fox (*Pteropus vampyrus*) in Malaysia (Epstein et al., *In Press*). However, some regional populations of Brazilian free-tailed bats do not engage in long-distance seasonal migrations, although seasonal short-distance movements are suspected between summer maternity roosts and winter hibernacula. Although controlled studies have not been conducted, stable insect abundance in the southeastern and northwestern parts of the Brazilian free-tailed bat's geographic range may support regional philopatry. Alternatively, the range expansion of this species into the southeastern and northwestern regions, and associated behavioral shifts in migration tendency, may have resulted from successful adaptation to use of building roosts (Davis et al., 1962). Local and long-distance migration of bats (Constantine, 1967b), and seasonality in general (Altizer et al., 2006; Nelson et al., 2002), are expected to have strong effects on epizootiological processes. The migration of infectious disease reservoir hosts is also a public health concern in regard to prevention and control efforts.

In bridge colonies of Brazilian free-tailed bats across the southern US, seasonal patterns of VNA seroprevalence are similar during the Early and Mid periods in both regions (30-50%), but seroprevalence was significantly higher in colonies from Texas compared to the southeastern US during the Late period among adult (Figure 3.4) and juvenile bats (Figure 3.6). Seasonal changes in colony composition and long-distance

autumn migrations may increase susceptibility to RABV infection, in part due to energetic costs associated with flight, vulnerability to cooler weather conditions that are presumed to initiate the autumn migration (Herreid, 1963; Villa-R and Cockrum, 1962), and perhaps lower insect availability in the fall months. Higher VNA seroprevalence in adult and juvenile Brazilian free-tailed bats from Texas colonies during August (i.e. Late period) suggests that higher RABV infection pressure may relate to differences in migratory behavior of bats. Although bats in Texas are not presumed to initiate autumn migration until late September and early October, there may be physiological stress associated with preparation for this event. The body condition of adult male, adult female, and juvenile bats was higher in Florida bridge colonies compared to Texas colonies during the Late period (Figure 3.8, Figure 3.10, Figure 3.12), suggesting that prey resources may be lower in Texas during the late summer months, leading to high intraspecific competition among bats and greater physiological stress prior to the autumn migration. It is also known that adult females tend to abandon the maternity colonies as they transition into a non-reproductive state, perhaps to escape high intraspecific competition as volant juvenile bats begin to compete for similar local prey resources (Constantine, 1967a; Davis et al., 1962). This may have altered colony composition in Texas roosts, and associated sampling for body condition and rabies VNA, during mid to late August. There have not been studies that address whether the proportion of antagonistic interactions or contact structure among roost-mates changes over time in relation to colony composition during this period, likely because tracking individually-marked bats is extremely difficult in massive colonies and when individuals are capable of traveling long distances over short periods of time. Very little is known

about the seasonal movements of Brazilian free-tailed bats in the southeastern US. Most bats are not present in the northern sites sampled in the southeastern US during the winter months (QBH, TCB, SACB, BCB), although site fidelity at these roosts across years was observed (see Chapter 2). Future studies should address whether regional observations on infection pressure (i.e. VNA seroprevalence) are consistent across years, and include sampling that extends further into the autumn months to improve hypothesis testing associated with variation in RABV epizootiology, energetic stress, and immune function, in relation to migratory behavior of temperate zone bats in the US.

Life History

Seasonal reproductive activity of adult female bats has important effects on body condition and rabies VNA seroprevalence at local scales. VNA seroprevalence among adult females significantly fluctuated among cave colonies in Texas, and bridge roosts in the southeastern US, and was consistently higher during lactation (Figure 1.3, Figure 2.4), at a time when females may be more susceptible to disease, due to poor body condition, relatively high contact rates with young, and potentially higher levels of parasitism (Pearce and O'Shea, 2007; Turmelle, 2005). Although a drop in body condition was expected among females following parturition (loss of fetal weight), it was unexpected that female body condition would not recover in transitioning from lactational to non-reproductive states, particularly for females in Texas that must prepare for return migration to Mexico (Figure 3.7, Figure 3.8). As previous studies have demonstrated elevated levels of RABV infection in pups (Constantine, 1986;

Steece and Altenbach, 1989), they may contribute to higher infection pressure observed in lactating adult females. The significance of maternally transmitted antibody in protecting pups against infection in otherwise pathogen-rich environments (i.e. caves) is poorly understood. As mentioned in a previous chapter, comparative susceptibility to RABV infection among adult and juvenile cohorts has not been experimentally tested, although it may be important with regard to aerosol exposure in roosts with massive aggregations of bats.

The seasonal life history schedules of adult female bats may also affect body condition and potential disease susceptibility in male cohorts. Particularly at roosts where reproductively active adult females are dominant, including almost all caves and some bridges, males could be at a serious disadvantage due to strong competition for resources during times when females have peak energetic demands (Kunz et al., 1995), and also due to greater contact with large clusters of susceptible young during the female reproductive season compared to roosts where sex ratios are closer to equal. Consistent with these hypotheses, male bats were largely absent from most cave colonies, and appeared to have significantly lower body condition during female pregnancy at most roosts across regions. In Texas, males that inhabited bridge roosts had stable body condition during female pregnancy, but at caves, and southeastern bridge and bat house roosts, male body condition was significantly lower during female pregnancy (i.e. Early period). Furthermore, males were significantly more likely to be seropositive among the southeastern colonies, but not among Texas (mostly bridge) colonies. These data provide suggestive, but not conclusive, evidence on how body condition may contribute to susceptibility to RABV infection. However, the model testing

does not support inclusion of body condition as a predictor in the best models of VNA seroprevalence.

Roost Ecology

Roost ecology appears to have a stronger influence on local patterns of rabies VNA seroprevalence, but the effect may weaken at landscape scales. Among Texas colonies, roost type was not a predictor of VNA seroprevalence, but did impact the patterns observed during the reproductive season. Among southeastern colonies, roost type was a predictor of rabies VNA seroporevalence, where bat house colonies had higher estimates overall. At the landscape scale, roost type was an important predictor only during the Early period, where bat house colonies exhibited significantly high levels of VNA seroprevalence compared to bridge or cave colonies. In the southeastern US, males were more likely to be seropositive, a result that may relate to the potential for greater antagonistic interactions, particularly during the mating period among male bats. In a Texas bridge colony of Brazilian free-tailed bats, two different copulation strategies were used by males, passive and aggressive, with the aggressive strategy (i.e. resource defense polygyny) used more frequently among smaller clusters of female bats (Keeley and Keeley, 2004). Given that the colony sizes in the southeastern US are generally smaller, and females are not nearly as dominant in the region, aggressive mating behaviors among males may be more common than observed in Texas, and lead to high contact rates among and between the sexes in mid to late March. Future studies on the impacts of roost type, colony size, and gender ratio, on behavior and contact rates during mating and across the reproductive season are critical for greater insight

into RABV exposure, and for characterization of dynamic contact networks for bat epizootiological models. Alternatively, bat house colonies may be subject to higher RABV infection pressure compared to bats in other types of roosts. Higher VNA seroprevalence could also result from a greater proportion of immunocompetent bats that are able to mount strong humoral (i.e. VNA) response to RABV infection in bat house colonies. Clearly, additional study is needed to address seasonal relationships between VNA seroprevalence estimates, contact rates, immune competence and RABV infection pressure among bat colonies in different roosts.

Variation in roost ecology for Brazilian free-tailed bats in the southwestern US is known to influence average colony size and gender ratios across sites, where caves tend to have larger colonies of bats and significantly higher proportions of females (Constantine, 1967a; Davis et al., 1962; Turmelle et al., *In Press*). In the southeastern US, only one (bridge) site had a high proportion of females, and gender ratios otherwise were close to equal across sites. Similarly, large (>1,000) and small (200-500) colonies were found with equal frequency among bridge and bat house roosts in the southeastern US. Thus, we might predict the impact of roost type per se to be lower in the southeastern US, but it was a stronger predictor of VNA seroprevalence than in Texas. Although roost type appears to impact the body condition of adult female bats across the landscape (Figure 3.7), effects on males and juvenile bats were not significant (Figure 3.9, Figure 3.11). Thus, although geographic region appears to be a more consistent predictor of variation at the landscape scale, future studies should consider even replication of multiple roost types across regions to simultaneously evaluate the influence of local and landscape factors.

It has been shown that the presence of multiple interacting disease reservoirs can lead to higher levels of infection (Craft et al., 2008; Guerra et al., 2003). For regional comparisons among bridge roosts, we might predict that if the presence of multiple bat species positively affected the force of infection, then Brazilian free-tailed bats in the southeastern US would have higher VNA seroprevalence compared to colonies in Texas. However, there are many complicating factors that may obscure the effects of multi-species interactions on RABV infection in bats. Future studies should include adequate replication of single or mixed-species colonies of bats, while controlling for year, roost type, region, and colony size. Furthermore, surveillance across all species in a colony would aid in evaluating variation in the force of infection within a roost across different species. Although significantly higher VNA seroprevalence was not observed among the mixed species colonies examined in this study, the species of bat in the roost may be a very important factor in determining the likelihood of cross-species transmission (Streicker et al., *In Prep.*).

Mortality pressures may also vary substantially for colonies of Brazilian free-tailed bats in Texas compared to colonies in Georgia and Florida. As has been documented previously (Constantine, 1967a; Davis et al., 1962), massive accumulations of dermestid beetles were observed on the floors of cave roosts, which rapidly reduce fallen bats to skeletal remains. Groups of cockroaches and ants were also commonly observed aggregating on and consuming fallen pups underneath the largest bridge (MB) colony sampled in Texas. There are also larger predators at cave roosts, including raccoons, skunks, foxes, and ringtails, all of which scavenge on bats that may have collided and fallen at the entrance to the cave during the evening

emergence (Davis et al., 1962; Kunz and Robson, 1995; Winkler and Adams, 1972). Mammalian predators and groups of scavenging insects were rarely encountered in proximity to southeastern colonies, although low numbers of avian predators were observed. This is probably due to the smaller colony sizes of bats in the southeastern US. Studies have also reported population die-offs resulting from toxic levels of agricultural pesticides in Brazilian free-tailed bats (Clark, 2001; Geluso et al., 1976), although both geographic regions have substantial agricultural activity. Comparative proportions of bats with pesticide residue were not obtained, but may be important predictors of disease susceptibility. Although links with mortality are not well understood, the body condition of juvenile bats in the southeastern US was significantly higher compared to bats in Texas, and the VNA seroprevalence among the juvenile cohort of Texas was significantly greater, although neither juvenile cohort had significantly different VNA seroprevalence compared to their respective adult cohorts during the Late period. RABV infection and mortality pressure appear to be greater for the Texas colonies during the sampling period, although additional study is warranted to document whether patterns and trends vary across years.

Closing Observations and Recommendations

We expect elevated infection pressure resulting from annual pulses of highly susceptible young following parturition, coupled with contact rates that may increase by orders of magnitude during lactation, or during migration periods when contact rates and energetic demands are high, but it is difficult to accurately measure such changes in the force of RABV infection with oral swab or VNA seroprevalence data alone.

Although the use of oral swabs to detect viral RNA is a minimally invasive technique that has become increasingly popular, few data exist to document the sensitivity of this technique for use in bats. Particularly among bats that have been experimentally infected, excretion of rabies virus in the saliva of bats appears to be a rare and transient phenomenon, although it is possible that captive environments may lead to altered infection dynamics. The low number of positive swabs encountered in this study is consistent with presumed levels of RABV infection in bats, but also preclude a robust comparative analysis with regard to other factors of concern in the study. Furthermore, rabies VNA titers tend to be dynamic through time in bats with known exposure history, and may wane to levels that are considered 'negative,' although the bat may have been infected in the past and may still have immunological memory (Amengual et al., 2007; Jackson et al., 2008). Thus, VNA seroprevalence estimates do not precisely differentiate among proportions of recently infected, previously infected, or susceptible cohorts of bats in the wild, but likely provide an estimate of relative infection pressure. Additional data are needed to characterize seasonal heterogeneities in the immune competence among different age and gender cohorts of bats, and would aid in testing predictions from models that suggest the importance of host heterogeneity for RABV epizootiology (Dimitrov and Hallam, 2009; Dimitrov et al., 2008; Dimitrov et al., 2007). Data on variation in bat contact rates are critically needed, and how these vary by gender, species, roost ecology, region, and life history.

One of the greatest difficulties for current and future studies of bat lyssavirus epizootiology will be estimating variation in infection pressure at local scales. Systematic sacrificing of hundreds of bats at multiple roosts is undesirable, given that

multiple bat species are already in decline in North America due to a variety of causes [e.g. wind turbine strikes, White Nose Syndrome (Blehert et al., 2008), habitat loss, etc.]. It will be critical to find effective, yet minimally invasive, techniques to investigate ecological factors associated with contact rates, susceptibility, and infection prevalence in natural populations in order to estimate and predict the force of infection in epizootiological disease models in bats.

Chapter 4

Comparative pathogenicity of rabies viruses in big brown bats (*Eptesicus fuscus*)

Introduction

The structure of human and wildlife populations across the landscape has strong implications for the dynamics and emergence of infectious diseases (Jones et al., 2008; Real and Biek, 2007). Major developments have been made in theoretical (Boots et al., 2004; Boots and Sasaki, 1999, 2000; Cross et al., 2005; Haraguchi and Sasaki, 2000; Rand et al., 1995; Webb et al., 2007) and experimental (Boots and Mealor, 2007; Bull et al., 1991; Kerr et al., 2006; Messenger et al., 1999) models linking population structure, disease dynamics, and pathogen evolution, with particular advances in our understanding of how contact networks regulate pathogen persistence and virulence. Evidence from natural systems also has demonstrated ecological relevance of theories on spatial infection processes (de Roode et al., 2008; Ewald, 1991; Herre, 1993). Bats exhibit a wide range of morphological, ecological, and behavioral characteristics, and similarly, are characterized by highly variable patterns of population structure (Burland and Worthington-Wilmer, 2001; Nowak, 1999). These features make the Order Chiroptera ideal for testing hypotheses regarding patterns of host-pathogen co-evolution.

Big brown bats (*Eptesicus fuscus*) are widely distributed and common throughout much of North America (Figure 4.1). They are habitat and foraging generalists (Kurta

and Baker, 1990; Sullivan et al., 2006) with short distance seasonal dispersal (Davis et al., 1968; Mills et al., 1975). Typical flight distances between roosts and to foraging grounds have been estimated to be as little as 1-2 km (Kurta and Baker, 1990), and seasonal aggregations in summer and winter roosts are rarely greater than 80 km apart (Beer, 1955; Goehring, 1972; Mills et al., 1975; Neubaum et al., 2006). Consistent with the low levels of dispersal observed in this species, the population genetic structure of big brown bats demonstrates highly divergent regional mitochondrial DNA (mtDNA) lineages in North America, with variable lineage sub-structuring within regional populations (Figure 4.2) (Turmelle et al., *In Prep.*), and a potential contact zone between regional populations in Colorado (Neubaum et al., 2007). Similar phylogenetic structure has been observed for *E. fuscus* RABV (Davis et al., 2006; Messenger et al., 2003b; Smith, 1996; Streicker et al., *In Prep.*), although strict correspondence between regional mitochondrial lineages and RABV variants of the host was not found in the apparent contact zone in Colorado (Neubaum et al., 2008).

Big brown bats consistently rank highest in the numbers of bats submitted for rabies testing each year among the 45 species of bats in the United States (US) (Mondul et al., 2003). However, this species has been implicated in only 3% (1 of 36) of indigenous human rabies cases in the US since 1980 (Blanton et al., 2008; Messenger et al., 2002). Despite the rare incidence of human RABV infection associated with this species, big brown bats have been implicated in cross-species transmission of RABV to terrestrial carnivores and other bats in the US (Leslie et al., 2006; Messenger et al., 2003b; Shankar et al., 2005). Despite evidence of distinct regional epizootiology and population genetic structure of the host, there are no published studies comparing

pathogenicity of *E. fuscus* RABV across the geographic range of this important reservoir.

This study compares the pathogenicity of eastern and western isolates of *E. fuscus* RABV for experimentally infected eastern big brown bats, and evaluates the effect of viral dose on survival within and across isolates. Given previous experimental infections with bats (Baer and Bales, 1967; Constantine, 1966; Constantine and Woodall, 1966), we predict a positive relationship between RABV dose and pathogenicity in experimentally infected bats for either isolate. When controlling for viral dose, we expected the western isolate of *E. fuscus* RABV to be more pathogenic compared to the eastern isolate, due to evidence of geographically isolated infection cycles and a greater likelihood for immune system recognition of and response to the eastern isolate in eastern big brown bats.

Methods

Animal Sampling

The experiments cited in this study were performed with 103 wild-caught big brown bats (*E. fuscus*) from building colonies across Georgia. All capture, handling, and experimental procedures were compliant with the Centers for Disease Control Institutional Animal Care and Use Committee. Collection of animals from the wild was performed under Georgia permits #29-WSF-05-14 and #29-WMB-01-129. Bats were individually marked with metal forearm bands or ear tags, and held captive in quarantine for a minimum of one month. Baseline diagnostics were performed, that assay for the presence of rabies virus neutralizing antibodies (VNA) in blood serum of bats and RABV

RNA on oropharyngeal swab samples from bats, to confirm naïve status of animals prior to experimental treatments. During quarantine and experimental treatments, bats were held in groups of three to six animals in stainless steel cages that measure 813 mm X 305 mm X 254 mm. All cages were collectively housed in a room at 75-80ºF and 30-50% humidity.

Viral Isolation and Characterization

The viral isolates used in the study were collected from the salivary glands of two naturally infected big brown bats: one from Colorado in 2004 (COef50; A04-0719) and one from Pennsylvania in 2006 (PAef137; A06-3684). The salivary gland homogenates were passaged once in mouse neuroblastoma (MNA) cell culture. The COef50 inoculum titrated to $10^{4.5}$ median mouse intracerebral doses per ml ($MICLD_{50}$/ml), and the PAef137 inoculum titrated to $10^{6.2}$ MICLD50/ml.

The viral isolates were characterized by sequencing and comparison in a phylogenetic analysis to an independent sample of RABV sequence data from big brown bats across the US. Parameters for the model of sequence evolution were estimated using MODELTEST (Posada and Crandall, 1998), and AIC values indicated that the K81+G model provided the best fit to the data (AIC=1824.2, K=3, -lnL=909.1). Markov Chain Monte Carlo (MCMC) simulations were run in a Bayesian framework for 2.4 million generations, sampling every 1,000 generations (burn-in=200) using MR. BAYES V.3.1 (Ronquist and Huelsenbeck, 2003). Posterior probabilities represent the group frequencies (from a consensus of 2002 trees) for nodes of interest (Figure 4.3).

Experimental Treatments

On Day 0 (actual dates vary by experiment), bats were randomly selected from cages and inoculated with 25 ul of varying doses of RABV in both left and right masseter muscles (total volume = 50 ul). For the COef50 isolate, inoculated doses were $10^{1.1}$, $10^{1.8}$, $10^{2.5}$, or $10^{3.2}$ $MICLD_{50}$s, after correcting for injection volume. For the PAef137 isolate, inoculated doses were $10^{-0.1}$, $10^{0.9}$, $10^{1.9}$, $10^{2.9}$, $10^{3.9}$, or $10^{4.9}$ $MICLD_{50}$s, also corrected for injection volume. All animals were observed daily for 140 days post-infection. Blood serum and oropharyngeal swab samples were taken from all bats, typically weekly for the first month post-infection, and bi-weekly or monthly for the remainder of an experiment (data not shown).

Diagnosis of RABV infection

Upon presentation of two or more clinical signs of rabies infection (i.e. paresis, paralysis, ataxia, atypical aggressive or reclusive behavior), bats were euthanized by intramuscular sedation with ketamine hydrochloride, intracardiac exsanguination under heavy sedation, and intracardiac injection of a barbituate (pentobarbital sodium and phenytoin sodium). Necropsy was performed on all euthanized bats, and brain tissue was removed aseptically. Brain stem tissue impressions were made on glass slides and fixed in acetone at -20°C. RABV antigen was detected and visualized by the direct fluorescent antibody test (dFA) on fixed brain stem impressions (Dean et al., 1996), using fluorescein isothiocyanate (FITC)-labeled monoclonal antibody (mAb) conjugate (Fujirebio Diagnostics, Inc., Malvern, PA).

Detection of viral RNA in saliva

Oropharyngeal samples were collected on paired sterile polyester swabs soaked in minimal essential medium (MEM-10). One swab was immediately placed in one ml of TRIzol® (Invitrogen Corporation, Carlsbad, CA), and stored at -80°C until testing, and the other swab was placed in one ml of MEM-10 for potential viral isolation. Viral RNA was extracted from the swab samples fixed in TRIzol®, and the reverse transcriptase polymerase chain reaction (RT-PCR) technique was used to attempt amplification of viral RNA from salivary samples as described previously (Jackson et al., 2008; Orciari et al., 2001).

Detection of RABV neutralizing antibodies

A modified rapid fluorescent focus inhibition test (RFFIT) (Jackson et al., 2008; Smith et al., 1996), using rabies challenge virus standard (CVS-11, V399) (Briggs et al., 1998), was used to assay for RABV-specific viral neutralizing antibodies (VNA) in the blood plasma of individual bats. The lowest bat plasma dilution tested in the RFFIT assay was 1:4, and sequential 2-fold dilutions were tested up to 1:2048. Rabies VNA endpoint titers of individual bats were calculated (Reed and Muench, 1938), and were converted to international units (IU/ml) by comparison to a control standard rabies immune globulin (SRIG) containing 2 IU/ml. Final titers of less than 0.06 IU/ml were considered negative for rabies VNA. Positive VNA titers (≥0.06 IU/ml) were interpreted as being indicative of prior RABV exposure. The choice of this cutoff value follows

previous studies for lyssavirus surveillance using bat and non-bat sera (Blanton et al., 2007; Jackson et al., 2008; Lumlertdacha et al., 2005; Rupprecht et al., 2005; Shankar et al., 2004). A previous study has demonstrated that the immunoglobulin G (IgG) fraction of the bat serum is responsible for neutralization activity against RABV (Shankar et al., 2004).

Statistical Analyses

Six animals with positive VNA titers prior to experimental inoculation were not included in the analyses (remaining n=97). All statistical analyses were performed using SAS v.9.1 (SAS Institute, Inc., Cary, NC). A survival analysis was conducted (PROC LIFEREG), which estimates a survivorship curve function for the duration of the experiment, and accounts for censored data (i.e. animals that die during experiment, but without clinical RABV infection). In this analysis, all survivors were censored at Day 140. Initially, two survival curves were estimated for each isolate, one with dose treated as a categorical variable in the model and a second with dose treated as a continuous variable in the model. Data were also combined across isolates, and survival curves were re-fitted with dose as a categorical or a continuous variable. Lastly, survival curves were fit for each viral isolate (COef50, PAef137), with and without dose as a nested effect in the model. The likelihood χ_2 statistic was used to independently evaluate the significance of dose effects within inocula, and isolate effects between inocula (α=0.05).

A nested mixed logistic regression (PROC GLIMMIX) also was performed, with dFA result treated as a binomial response variable (0=survivor on Day 140, 1=RABV infection of CNS before or on Day 140). This model cannot account for non-survivor censored data (i.e. bats that died before Day 140, but were not dFA positive), and the regression was performed for a subset of bats that excluded such animals. Only experiments associated with the PAef137 isolate had censored animals. Excluding two animals that were excluded due to positive baseline VNA titers (bat #21 and bat #483), three additional bats with non-specific deaths on Days 43, 113, 122, were excluded prior to the mixed logistic regression analyses. While this type of analysis does not estimate survivorship curves as a function of time explicitly, it allowed for certain factors to be treated as random effects. It was appropriate to check the influence of random effects, as dose treatments were part of the replication treatment for each viral isolate, but actual doses were not identical across isolates.

Lastly, nonparametric analyses were used to test for differences in the incubation periods across isolates and doses. Spearman's Rank Order Correlation test was performed to evaluate the effects of dose, as a continuous variable, on the incubation periods of animals that succumbed to infection. Also, a nonparametric ANOVA model was tested (PROC NPAR1WAY) to evaluate the effects of dose, as a categorical variable, on the incubation period of animals that succumbed to infection, and in a separate model to evaluate the effects of viral isolate on the incubation period of animals that succumbed to infection (α=0.05).

Results

Pathogenicity - Dose

In the survival analyses, categorical dose effects were not significant for the COef50 isolate (**n=41, df=3, 37, χ_2=4.52, p=0.21**; Table 4.1, Figure 4.4). In the survival analysis with dose treated as a continuous predictor, dose effects also were not significant (**n=41, df=1, 39, χ_2=2.60, p=0.11**). A logistic regression model with categorical dose as predictor of dFA result on uncensored bats gave a similar result (**n=41, df=3, 37, F=1.71, p=0.18**). A logistic regression model with dose as a continuous predictor of dFA result also was not significant (**n=41, df=1, 39, F=1.92, p=0.17**). There was not a significant correlation between dose, treated as a continuous variable, and incubation period among bats succumbing to infection (**n=31, Spearman's ρ=(-0.22), p=0.24**), and a nonparametric ANOVA model with dose as a categorical predictor of incubation period was not significant (**n=31, df=3, 27, F=0.48, p=0.70**).

Categorical dose effects were not significant in the survival analysis for the PAef137 isolate (**n=56, df=5, 50, χ_2=3.04, p=0.69**; Table 4.2, Figure 4.5). In the survival analysis with dose as a continuous predictor, dose effects were also not significant (**n=56, df=1, 54, χ_2=0.0001, p=0.99**). A logistic regression model of categorical dose effects on dFA result for the uncensored subset of bats was not significant (**n=53, df=5, 47, F=0.67, p=0.65**), nor was a logistic regression with dose treated as a continuous predictor of dFA result (**n=53, df=1, 51, F=0.05, p=0.82**). There was a marginally significant correlation between continuous dose and incubation period for bats succumbing to infection (i.e. dFA positive) (**n=24, Spearman's ρ=(-0.36), p=0.08**), and

a nonparametric ANOVA model with dose as a categorical predictor of incubation period was marginally significant (**n=24, df=4, 19, F=2.64, p=0.07**).

Across both isolates, a significant effect of categorical dose was detected in the survival analysis **(n=97, df=9, 87, χ_2=21.3, p=0.01)**, with higher doses associated with reduced survivorship. In the survival analysis with dose treated as a continuous predictor, dose effects were not significant (**n=97, df=1, 95, χ_2=0.50, p=0.48**). A logistic regression on the uncensored subset of bats did not recover a significant effect of categorical dose on dFA result across isolates (**n=94, df=9, 84, F=1.56, p=0.14**). The logistic regression of continuous dose as a predictor of dFA result was not significant (**n=94, df=1, 92, F=0.73, p=0.39**). There was a marginally significant correlation between continuous dose and incubation period for bats succumbing to infection (i.e. dFA positive) (**n=55, Spearman's ρ=(-0.25), p=0.07**), and nonparametric ANOVA model of categorical dose effects on incubation period was significant (**n=55, df=8, 46, F=3.60, p=0.003**).

Pathogenicity - Virus

In the survival analyses comparing the viral isolates, an effect of viral isolate was detected (**n=97, df=1,95, χ_2=14.8, p<0.0001**; Figure 4.6), with the COef50 isolate significantly more pathogenic. In a survival analysis, with dose as a continuous predictor nested within virus, the effect of viral isolate was marginally significant (**n=97, df=1, 93, χ_2=3.08, p=0.08**; dose [virus] χ_2=2.51, p=0.29). The survival analyses estimated different mean incubation periods for each isolate, and the mean for the PAef137 isolate

(97 ± 8 days) was three times as long as the mean incubation period for the COef50 isolate (29 ± 3 days).

In the nested mixed logistic regression analysis performed on the uncensored subset of bats, and including categorical dose as a random effect nested within virus, the effect of viral isolate on dFA result was marginally significant (**n=94; df=1, 8, F=4.95, p=0.06;** dose [virus]=0.30 [±0.43]), with the COef50 isolate more pathogenic. With continuous dose as a random effect nested within virus, the effect of viral isolate on dFA result was highly significant (**n=94, df=1, 90, F=8.31, p=0.005**; dose [virus]=1.12e-11). The nonparametric ANOVA model of viral isolate effects on incubation periods of bats succumbing to infection was highly significant (**n=58, df=1, 56, F=8.85, p=0.004**), with significantly shorter incubation periods for bats infected with the COef50 isolate.

Discussion

Previous studies have demonstrated that RABV infection cycles in bats occur primarily via intraspecific transmission. If host population structure is nonrandom across the landscape or geographic range, RABV epizootiology should also be non-random in space and time, and may significantly impact subsequent viral evolution. As existing studies of big brown bat population structure and RABV lineage structure represent independent samples of animals, statistical evaluation of the spatial association between host and virus is not possible in the current study. However, the geographic consistency of both host and viral structure suggests spatially structured RABV epizootiology (Figure 4.2, Figure 4.3), and demonstrates that host population structure

can influence epizootiological substructure and viral richness in a bat reservoir (Davis et al., 2006; Franka et al., 2006; Hughes et al., 2005; Messenger et al., 2003b; Smith, 1996; Velasco-Villa et al., 2006). In this study, we have also demonstrated that RABV isolates from genetically divergent regional populations of big brown bats may also differ in pathogenicity.

Cross-species transmission of RABV among bats (Shankar et al., 2005), and from bats to terrestrial carnivores and humans (Leslie et al., 2006; Messenger et al., 2003b), suggests altered pathogenicity of some bat RABVs. However, variation among bat RABV variants implicated in rabies cases among terrestrial carnivores in the US is consistent with common bat RABV variants in certain geographic areas (Messenger et al., 2003b). Although human rabies cases in the US suggest a disproportionate association with silver-haired bat (*Lasionycteris noctivagans*) and eastern pipistrelle (*Perimyotis subflavus*) bat RABV variants, there are few comparative estimates of variable pathogenicity across bat isolates. One study found that a RABV isolate associated with silver-haired bats could replicate more effectively at lower temperatures and in peripheral (epithelial) tissues compared to a coyote RABV isolate (Morimoto et al., 1996), but this may be an adaptation present in other bat RABVs. Another study suggested that silver-haired bat RABV was more pathogenic than a fixed laboratory strain of RABV, perhaps because it was better at evading the host innate and antiviral responses compared to the laboratory strain (Wang et al., 2005). Testing across multiple bat RABV isolates should be a priority for future comparative studies to provide more conclusive evidence as to whether some bat species harbor more pathogenic

RABV, or whether observed differences between bat and terrestrial carnivore or fixed laboratory strains of RABV are general features of most bat RABVs.

Inoculation dose of RABV was not a predictor of survival to infection in the current study, but did have marginal effects on the length of the incubation period among animals that developed clinical infection, which is consistent with previous studies (Baer and Bales, 1967). Although additive dose-dependence was not observed in either isolate, a threshold may exist whereby a bat has a marginal compared to a high probability of becoming clinically infected. From the current doses and isolates explored, this threshold appears to fall around 10^1 $MICLD_{50}$s (Figure 4.4, Figure 4.5). When dose was equal to or greater than 10^2 $MICLD_{50}$s, the survival curves appeared to stabilize across both isolates. However, we cannot exclude the possibility that some bats captured from the wild may have had previous RABV exposure, as evidenced by the bats with positive baseline VNA titers that were excluded from the analyses. It has been observed in this and other experiments that the absence of VNA does not provide definitive indication of naïve infection status (Jackson et al., 2008). Bats with negative baseline VNA may have previous RABV exposure, which may have altered the susceptibility of some bats and obscured dose-dependent relationships, or may lead to overestimation of apparent threshold doses.

Current data suggest that western big brown bat RABV may be more pathogenic than eastern big brown bat RABV, although only one isolate per region was tested. Better replication of representative isolates across regions and reciprocal testing on western big brown bats are needed to provide conclusive evidence for variable pathogenicity among regional big brown bat RABV isolates.

Conclusions

Variable pathogenicity was observed from intramuscular experimental infection of big brown bats with *E. fuscus* RABV isolates from eastern and western regions. Dose-dependent effects on survival probabilities were not detected for either isolate. However, comparisons across isolates revealed that higher doses were associated with shorter incubation periods in bats that develop clinical infection. As longer incubation periods in bats have been associated with higher viral titers of animals upon clinical disease presentation, there are likely to be negative feedbacks on the relationship between dose, incubation period, and infectious presentation. These data suggest that population genetic structure of a host may impact regional variation in RABV diversity and pathogenicity in big brown bats.

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APPENDICES

Appendix 1

Table 1.1 Proportion of adult Brazilian free-tailed bats with rabies VNA across six sites in south-central Texas. Data in bold were included in the statistical analyses.

Roost	Site	Period	Sex	N	Seroprevalence	Roost	Site	Period	Sex	N	Seroprevalence
Cave	FC	**Early**	**F**	**22**	**0.32**	Bridge	MB	**Early**	**F**	**17**	**0.53**
			M	**8**	**0.25**				**M**	**11**	**0.36**
		Mid	**F**	**24**	**0.33**			**Mid**	**F**	**21**	**0.38**
			M	**7**	**0.71**				**M**	**11**	**0.55**
		Late	**F**	**18**	**0.50**			**Late**	**F**	**15**	**0.47**
			M	**2**	**0.50**				**M**	**5**	**0.40**
		Pre-migratory	F	2	0.00			Pre-migratory	F	1	0.00
			M	2	0.50				M	-	-
	DBC	**Early**	**F**	**22**	**0.32**		SCB	**Early**	**F**	**3**	**1.00**
			M	7	0.43				**M**	**25**	**0.64**
		Mid	**F**	**23**	**0.74**			**Mid**	**F**	**8**	**0.50**
			M	-	-				**M**	**23**	**0.52**
		Late	**F**	**18**	**0.33**			**Late**	**F**	**12**	**0.75**
			M	-	-				**M**	**8**	**0.25**
		Pre-migratory	F	2	0.50			Pre-migratory	F	1	0.00
			M	-	-				M	7	0.14
	JRC	**Early**	**F**	**30**	**0.17**		EEB	**Early**	**F**	**11**	**0.18**
			M	3	0.00				**M**	**16**	**0.06**
		Mid	**F**	**28**	**0.36**			**Mid**	**F**	**7**	**0.57**
			M	-	-				**M**	**24**	**0.58**
		Late	**F**	**20**	**0.00**			**Late**	**F**	**6**	**0.33**
			M	-	-				**M**	**8**	**0.50**
		Pre-migratory	F	-	-			Pre-migratory	F	-	-
			M	-	-				M	-	-

Table 1.2 Proportion of juvenile Brazilian free-tailed bats with rabies VNA across six sites in south-central Texas. Data in bold were included in the analyses (n=50).

Roost	Site	Period	N	Seroprevalence	Roost	Site	Period	N	Seroprevalence
Cave	FC	Mid	-	-	*Bridge*	MB	Mid	-	-
		Late	**3**	**0.00**			**Late**	**4**	**0.50**
		Pre-migratory	**6**	**0.17**			**Pre-migratory**	**3**	**0.00**
	DBC	Mid	6	0.80		SCB	Mid	-	-
		Late	**1**	**0.00**			Late	-	-
		Pre-migratory	**9**	**0.56**			**Pre-migratory**	**6**	**0.50**
	JRC	Mid	-	-		EEB	Mid	-	-
		Late	**3**	**0.00**			**Late**	**15**	**0.47**
		Pre-migratory	-	-			Pre-migratory	-	-

Table 1.3 Logistic model output for VNA seroprevalence among adult Brazilian free-tailed bats from Texas.

Nested mixed logistic model – All Adults (n=463)

Class: Site, Roost, Period, Sex
Fixed: Roost, Period, Sex, Roost*Period, Sex*Period, Roost*Sex, Roost*Period*Sex
Random: Site [Roost]

Type 3 Effects†	df_n	df_d	$\chi 2$	F	Pr > $\chi 2$	Pr > F
Roost	1	4	0.62	0.62	0.43	0.48
Period	2	449	13.77	6.89	0.001	0.001
Sex	1	449	0.04	0.04	0.83	0.83
Roost*Period	2	449	6.07	3.03	0.05	0.05
Period*Sex	2	449	3.25	1.63	0.20	0.20
Roost*Sex	1	449	0.81	0.81	0.37	0.37

Covariance parameter
Site [Roost]: estimate=0.31, s.e.=0.26

†The Roost*Period*Sex interaction was removed from the final model (p=0.77).

Table 1.4 Logistic model output for VNA seroprevalence among adult Brazilian free-tailed bats from cave colonies in Texas.

Non-nested mixed logistic model – Cave Adults (n=232)

Class: Site, Period, Sex
Fixed: Period, Sex, Period*Sex — Random: Site

Type 3 Effects	df_n	df_d	$\chi 2$	F	Pr > $\chi 2$	Pr > F
Period	2	224	7.31	3.66	0.03	0.03
Sex	1	224	0.85	0.85	0.36	0.36
Period*Sex	2	224	1.05	0.52	0.59	0.59
Covariance parameter						
Site: estimate=0.47,	s.e=0.55					

Non-nested mixed logistic model – Cave Adult Females[§] (n=188)

Class: Site, Repc
Fixed: Repc — Random: Site

Type 3 Effects	df_n	df_d	$\chi 2$	F	Pr > $\chi 2$	Pr > F
Repc	2	183	3.70	1.85	0.16	0.16
Covariance parameter						
Site: estimate=0.44,	s.e=0.52					

[§]Females of undetermined reproductive status were excluded (n=17).

Non-nested mixed logistic model – Cave Adult Females (n=205)

Class: Site, Period
Fixed: Period — Random: Site

Type 3 Effects	df_n	df_d	$\chi 2$	F	Pr > $\chi 2$	Pr > F
Period	2	200	9.02	4.51	0.01	0.01
Covariance parameter						
Site: estimate=0.43,	s.e=0.50					

Non-nested mixed logistic model – Cave Adult Males (n=27)

Class: Site, Period
Fixed: Period — Random: Site

Type 3 Effects	df_n	df_d	$\chi 2$	F	Pr > $\chi 2$	Pr > F
Period	2	22	3.67	1.83	0.16	0.18
Covariance parameter						
Site: N/A, only one site - FC						

Table 1.5 Logistic model output for VNA seroprevalence among adult Brazilian free-tailed bats from bridge colonies in Texas.

Non-nested mixed logistic model – Bridges Adults (n=231)

Class: Site, Period, Sex
Fixed: Period, Sex, Period*Sex Random: Site

Type 3 Effects	df_n	df_d	$\chi 2$	F	Pr > $\chi 2$	Pr > F
Period	2	223	0.79	0.39	0.67	0.68
Sex	1	223	0.46	0.46	0.50	0.50
Period*Sex	2	223	2.66	1.33	0.26	0.27

Covariance parameter
Site: estimate=0.15, s.e.=0.21

Non-nested mixed logistic model – Bridge Adult Females[§] (n=99)

Class: Site, Repc
Fixed: Repc Random: Site

Type 3 Effects	df_n	df_d	$\chi 2$	F	Pr > $\chi 2$	Pr > F
Repc	2	94	2.10	1.05	0.35	0.35

Covariance parameter
Site: estimate=0.27, s.e=0.46

[§]Females of undetermined reproductive status were excluded (n=1).

Non-nested mixed logistic model – Bridge Adult Females (n=100)

Class: Site, Period
Fixed: Period Random: Site

Type 3 Effects	df_n	df_d	$\chi 2$	F	Pr > $\chi 2$	Pr > F
Period	2	95	0.42	0.21	0.81	0.81

Covariance parameter
Site: estimate=0.35, s.e=0.54

Non-nested mixed logistic model – Bridge Adult Males (n=131)

Class: Site, Period
Fixed: Period Random: Site

Type 3 Effects	df_n	df_d	$\chi 2$	F	Pr > $\chi 2$	Pr > F
Period	2	126	3.23	1.62	0.20	0.20

Covariance parameter
Site: estimate=0.04, s.e.=0.12

Table 1.6 Logistic model output for VNA seroprevalence among juvenile Brazilian free-tailed bats in Texas.

Nested mixed logistic model – Juveniles (n=50)						
Class: Site, Roost, Period						
Fixed: Roost, Period, BCI					Random: Site [Roost]	
Type 3 Effects	df_n	df_d	$\chi 2$	F	Pr > $\chi 2$	Pr > F
Period	1	42	1.96	1.96	0.16	0.17
Roost	1	4	0.99	0.99	0.32	0.38
BCI	1	42	2.77	2.77	0.10	0.10
Covariance parameter						
Site [Roost]: estimate=0.24, s.e.=0.73						

Non-nested mixed logistic model – Juveniles (n=50)						
Class: Site, Period						
Fixed: Period, BCI					Random: Site	
Type 3 Effects	df_n	df_d	$\chi 2$	F	Pr > $\chi 2$	Pr > F
Period	1	42	1.67	1.67	0.20	0.20
BCI	1	42	3.08	3.08	0.08	0.09
Covariance parameter						
Site: estimate=0.14, s.e.=0.54						

Table 1.7 Comparisons on VNA seroprevalence between adult and juvenile cohorts during the Late period in the Texas.

Nested mixed logistic model – Late (n=138)						
Class: Site, Roost, Age						
Fixed: Roost, Age					Random: Site [Roost]	
Type 3 Effects	df_n	df_d	$\chi 2$	F	Pr > $\chi 2$	Pr > F
Roost	1	4	2.20	2.20	0.14	0.21
Age	1	131	0.45	0.45	0.50	0.50
Covariance parameter						
Site [Roost]: estimate=0.93, s.e.=1.07						
The Roost*Age interaction term was removed, as the model did not converge with it included.						

Table 1.8 ANOVA model output for body condition of adult Brazilian free-tailed bats in Texas.

Nested mixed ANOVA model – All Adults (n=519)

Class: Site, Roost, Sex, Period
Fixed: Roost, Sex, Period, Roost*Period, Sex*Period, Roost*Sex, Roost*Sex*Period
Random: Site [Roost]

Type 3 Effects	df_n	df_d	F	Pr > F
Roost	1	15	5.84	0.03
Period	2	506	1.26	0.29
Sex	1	477	4.58	0.03
Roost*Period	2	506	0.14	0.87
Sex*Period	2	506	6.49	0.002
Roost*Sex	1	477	6.35	0.01
Roost*Sex*Period	2	505	5.25	0.006

Covariance parameter
Site [Roost]: z=0.75, p=0.23

Nested mixed ANOVA model – Adult Females[§] (n=331)

Class: Site, Roost, Repc
Fixed: Roost, Repc, Roost*Repc Random: Site [Roost]

Type 3 Effects	df_n	df_d	F	Pr > F
Roost	1	4.87	9.53	0.03
Repc	2	325	30.70	<0.0001
Repc*Roost	2	325	2.90	0.06

Covariance parameter
Site [Roost]: z=0.86, p=0.19

[§]Females of undetermined reproductive status were excluded (n=20).

Nested mixed ANOVA model – Adult Males (n=158)

Class: Site, Period, Roost
Fixed: Period, Roost, Roost*Period Random: Site [Roost]

Type 3 Effects	df_n	df_d	F	Pr > F
Roost	1	4.46	0.38	0.57
Period	2	150	2.62	0.08
Roost*Period	2	150	3.71	0.03

Covariance parameter
Site [Roost]: z=0.82, p=0.21

Table 1.9 ANOVA model output for body condition of juvenile Brazilian free-tailed bats in Texas.

Nested mixed ANOVA model - Juveniles (n=67)				
Class: Site, Period, Roost				
Fixed: Period, Roost, Roost*Period				Random: Site [Roost]
Type 3 Effects	df_n	df_d	F	Pr > F
Roost	1	3.05	0.03	0.87
Period	1	51.6	13.68	0.0005
Roost*Period	1	51.6	0.01	0.92
Covariance parameter				
Site [Roost]:	z=0.95, p=0.17			

Non-nested mixed ANOVA model - Juveniles (n=67)				
Class: Site, Period, Sex				
Fixed: Period, Sex, Sex*Period				Random: Site
Type 3 Effects	df_n	df_d	F	Pr > F
Sex	1	58	0.12	0.73
Period	1	58	13.68	<0.0001
Sex*Period	1	58	0.19	0.66
Covariance parameter				
Site: z=0.89, p=0.19				

Table 1.10 Comparisons of body condition and body size across adult and juvenile cohorts during the Late period.

Nested mixed ANOVA model – Late, Body Condition (n=179)				
Class: Site, Roost, Age				
Fixed: Roost, Age, Roost*Age				Random: Site [Roost]
Type 3 Effects	df_n	df_d	F	Pr > F
Roost	1	7.83	0.12	0.73
Age	1	163	126.47	<0.0001
Roost*Age	1	163	1.70	0.19
Covariance parameter				
Site [Roost]:	z=0.43, p=0.33			

Nested mixed ANOVA model – Late, Forearm Length (n=179)				
Class: Site, Roost, Age				
Fixed: Roost, Age, Roost*Age				Random: Site [Roost]
Type 3 Effects	df_n	df_d	F	Pr > F
Roost	1	175	0.01	0.93
Age	1	175	2.89	0.09
Roost*Age	1	175	0.29	0.59
Covariance parameter				
Site [Roost]:	0			

Table 1.11 Model test results VNA seroprevalence data from adult bats in Texas.

Model	Factors	AIC	AICC	K	I
1	rt, sex, pd, rt*sex, rt*pd, sex*pd, rt*sex*pd, site (rt)	607.63	608.85	42	7.98
2	rt, sex, pd, rt*sex, rt*pd, sex*pd, rt*sex*pd, bci, site (rt)	609.24	610.61	43	9.59
3	rt, sex, pd, rt*sex, rt*pd, sex*pd, site (rt)	604.08	605.02	30	4.43
4	rt, sex, pd, rt*sex, rt*pd, sex*pd, bci, site (rt)	605.66	606.74	31	6.01
5	rt, sex, pd, rt*sex, rt*pd, site (rt)	603.65	604.34	24	4.00
6	rt, sex, pd, rt*sex, rt*pd, bci, site (rt)	605.43	606.24	25	5.78
7	rt, sex, pd, rt*sex, sex*pd, site (rt)	606.24	606.93	24	6.59
8	rt, sex, pd, rt*sex, sex*pd, bci, site (rt)	607.51	608.32	25	7.86
9	rt, sex, pd, pd*sex, rt*pd, site (rt)	602.98	603.79	26	3.33
10	rt, sex, pd, pd*sex, rt*pd, bci, site (rt)	604.74	605.68	27	5.09
11	rt, sex, pd, pd*sex, site (rt)	604.99	605.58	20	5.34
12	rt, sex, pd, pd*sex, bci, site (rt)	606.47	607.16	21	6.82
13	rt, sex, pd, pd*rt, site (rt)	602.25	602.84	20	2.60
14	rt, sex, pd, pd*rt, bci, site (rt)	604.12	604.82	21	4.47
15	rt, sex, pd, sex*rt, site (rt)	602.86	603.35	18	3.21
16	rt, sex, pd, sex*rt, bci, site (rt)	604.34	604.93	19	4.69
17	**rt, sex, pd, site (rt)**	**601.50**	**601.9**	**14**	**1.85**
18	rt, sex, pd, bci, site (rt)	603.12	603.61	15	3.47
19	**rt, pd, rt*pd, site (rt)**	**600.3**	**600.79**	**18**	**0.65**
20	rt, pd, rt*pd, bci, site (rt)	602.21	602.8	19	2.56
21	**rt, pd, site (rt)**	**599.65**	**599.97**	**12**	**0.00**
22	**rt, pd, bci, site (rt)**	**601.38**	**601.78**	**13**	**1.73**
23	rt, sex, rt*sex, site (rt)	609.84	610.16	15	10.19
24	rt, sex, rt*sex, bci, site (rt)	610.41	610.8	16	10.76
25	rt, sex, site (rt)	608.09	608.33	11	8.44
26	rt, sex, bci, site (rt)	608.84	609.16	12	9.19
27	pd, sex, pd*sex, site	604.99	605.58	18	5.34
28	pd, sex, pd*sex, bci, site	606.47	607.16	19	6.82
29	**pd, sex, site**	**601.50**	**601.9**	**12**	**1.85**
30	pd, sex, bci, site	603.12	603.61	13	3.47
31	rt, site (rt)	606.26	606.44	9	6.61
32	rt, bci, site (rt)	607.23	607.48	10	7.58
33	sex, site	608.09	608.33	9	8.44
34	sex, bci, site	608.84	609.16	10	9.19
35	**pd, site**	**599.65**	**599.97**	**10**	**0.00**
36	**pd, bci, site**	**601.38**	**601.78**	**11**	**1.73**
37	site	606.26	606.44	7	6.61
38	site, bci	607.23	607.48	8	7.58

Table 1.12 Model test results for VNA seroprevalence data from juvenile bats in Texas.

Model	Factors	AICC	K	I
1	rt, sex, period, site (rt)	76.62	13	6.04
2	rt, sex, period, bci, site (rt)	75.77	14	5.19
3	rt, sex, site (rt)	73.79	11	3.21
4	rt, sex, bci, site (rt)	73.36	12	2.78
5	rt, period, site (rt)	74.23	11	3.65
6	rt, period, bci, site (rt)	73.27	12	2.69
7	sex, period, site	76.62	11	6.04
8	sex, period, bci, site	75.77	12	5.19
9	**rt, site (rt)**	**71.52**	**9**	**0.94**
10	**rt, bci, site (rt)**	**70.58**	**10**	**0**
11	period, site	74.23	9	3.65
12	period, bci, site	73.27	10	2.69
13	sex, site	73.79	9	3.21
14	sex, bci, site	72.83	10	2.25
15	**site**	**71.52**	**7**	**0.94**
16	**site, bci**	**70.58**	**8**	**0**

Table 1.13 Model test results for body condition data from adult bats in Texas.

Model	Factors	AIC	AICC	K	I
1	**rt, sex, pd, rt*sex, rt*pd, sex*pd, rt*sex*pd**	**-1047.8**	**-1047.8**	**36**	**1.6**
2	rt, sex, pd, rt*sex, rt*pd, sex*pd	-1045.2	-1045.2	24	4.2
3	**rt, sex, pd, rt*sex, rt*pd**	**-1048.9**	**-1048.9**	**18**	**0.5**
4	rt, sex, pd, rt*sex, sex*pd	-1047.2	-1047.1	18	2.2
5	rt, sex, pd, pd*sex, rt*pd	-1042.3	-1042.3	20	7.1
6	rt, sex, pd, pd*sex	-1045.4	-1045.4	14	4.0
7	**rt, sex, pd, pd*rt**	**-1049.4**	**-1049.4**	**14**	**0.0**
8	rt, sex, pd, sex*rt	-1043.5	-1043.5	12	5.9
9	rt, sex, pd	-1044.2	-1044.2	8	5.2
10	rt, pd, rt*pd	-1029.0	-1029.0	12	20.4
11	rt, pd	-1028.2	-1028.1	6	21.2
12	rt, sex, rt*sex	-1036.3	-1036.3	9	13.1
13	rt, sex	-1034.6	-1034.6	5	14.8
14	pd, sex, pd*sex	-1043.6	-1043.6	12	5.8
15	pd, sex	-1042.3	-1042.2	6	7.1
16	rt	-1023.0	-1023.0	3	26.4
17	sex	-1033.0	-1032.9	3	16.4
18	pd	-1030.4	-1030.4	4	19

Table 1.14 Model test results for body condition data from juvenile bats in Texas.

Model	Factors	AICC	K	I
1	rt, sex, pd, rt*sex, rt*pd, sex*pd, rt*sex*pd	-265.0	27	30.2
2	rt, sex, pd, rt*sex, rt*pd, sex*pd	-263.0	19	32.2
3	rt, sex, pd, rt*sex, rt*pd	-270.0	15	25.2
4	rt, sex, pd, rt*sex, sex*pd	-269.5	15	25.7
5	rt, sex, pd, rt*pd, sex*pd	-266.3	15	28.9
6	rt, sex, pd, rt*pd	-273.1	11	22.1
7	rt, sex, pd, rt*sex	-276.5	11	18.7
8	rt, sex, pd, sex*pd	-272.9	11	22.3
9	rt, sex, pd	-279.7	7	15.5
10	rt, pd, rt*pd	-281.2	9	14.0
11	rt, pd	-288.0	5	7.2
12	rt, sex, rt*sex	-276.1	9	19.1
13	rt, sex	-274.8	5	20.4
14	sex, pd, sex*pd	-280.0	9	15.2
15	sex, pd	-286.9	5	8.3
16	rt	-283.1	3	12.1
17	**pd**	**-295.2**	**3**	**0**
18	sex	-280.9	3	14.3

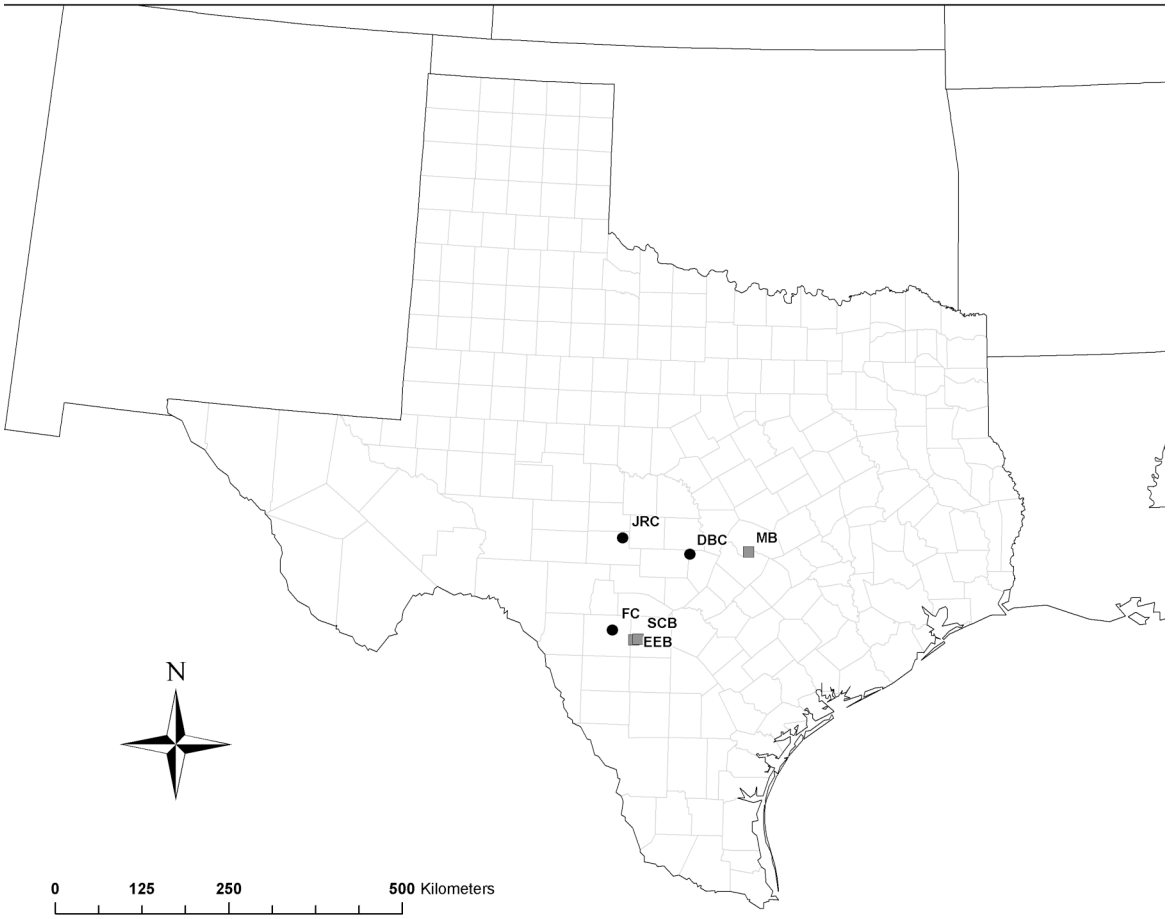

Figure 1.1 The geographic location of all sites sampled in Texas, USA. Caves colonies (black circles) include: Eckert James River Cave (JRC), Davis Blowout Cave (DBC), and Frio Cave (FC). Bridges colonies (gray squares) include: Seco Creek Bridge (SCB), East Elm Creek Bridge (EEB), and McNeil Bridge (MB).

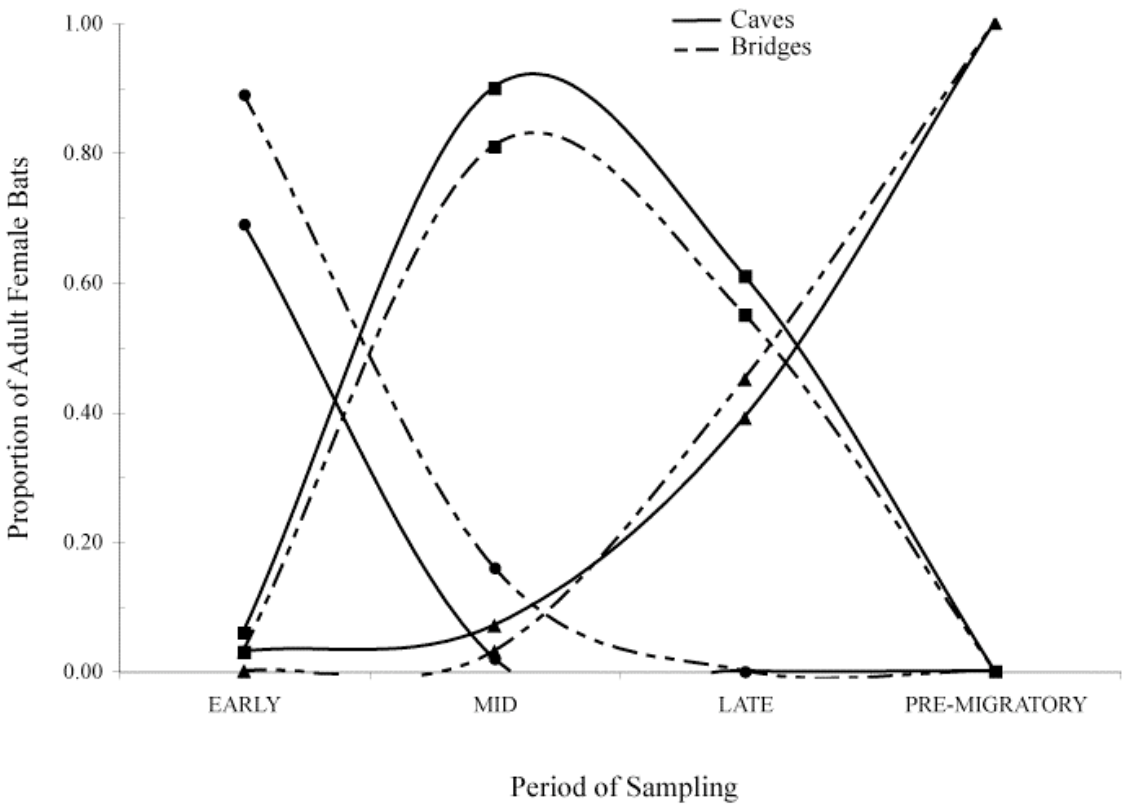

Figure 1.2 The reproductive activity of adult female bats measured during four periods: Early, Mid, Late, and Pre-migratory. Solid lines represent cave colonies, and dashed lines represent bridge colonies. Reproductive status was: pregnant (circle), lactating (square), or non-reproductive (triangle). Females of undetermined reproductive status are not shown (n=20).

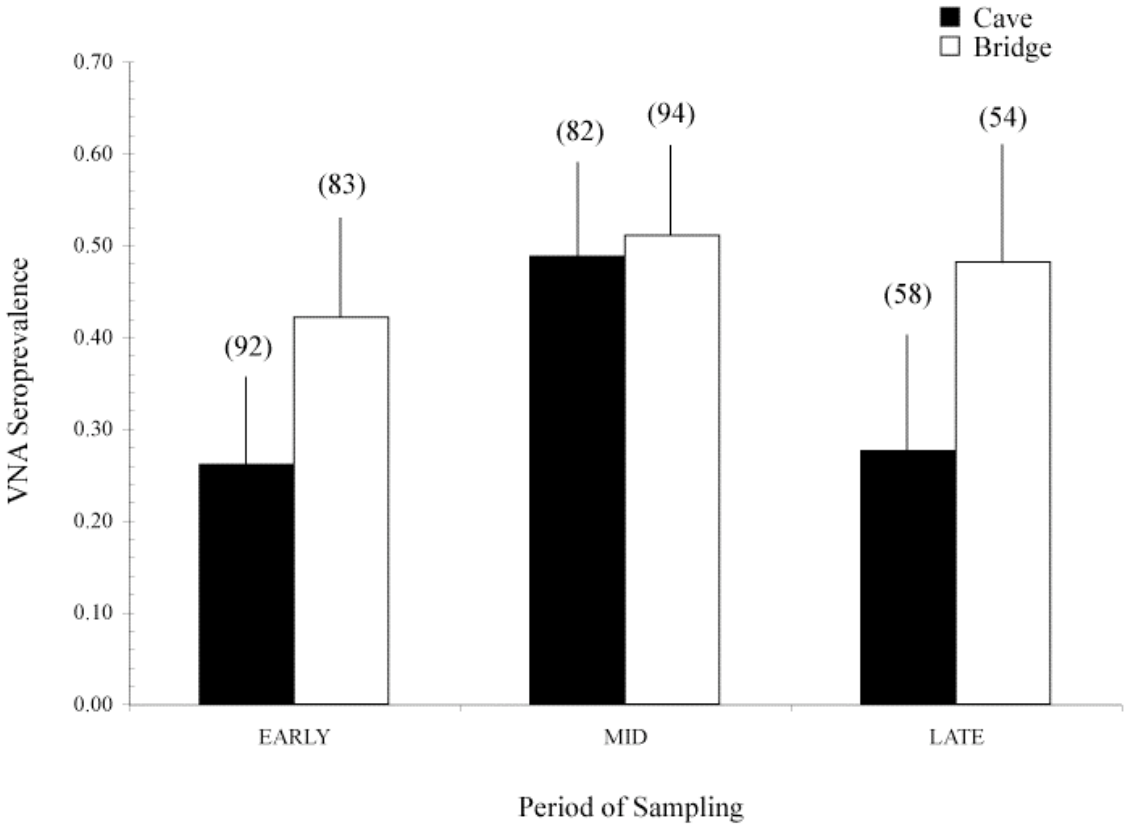

Figure 1.3 Mean VNA seroprevalence among colonies of adult bats, from three cave (black; FC, DBC, JRC) and three bridge (white; MB, SCB, EEB) colonies, across time periods (N=463). Upper 95% confidence intervals on proportions are shown above histogram bars, and sample sizes are included parenthetically above confidence intervals.

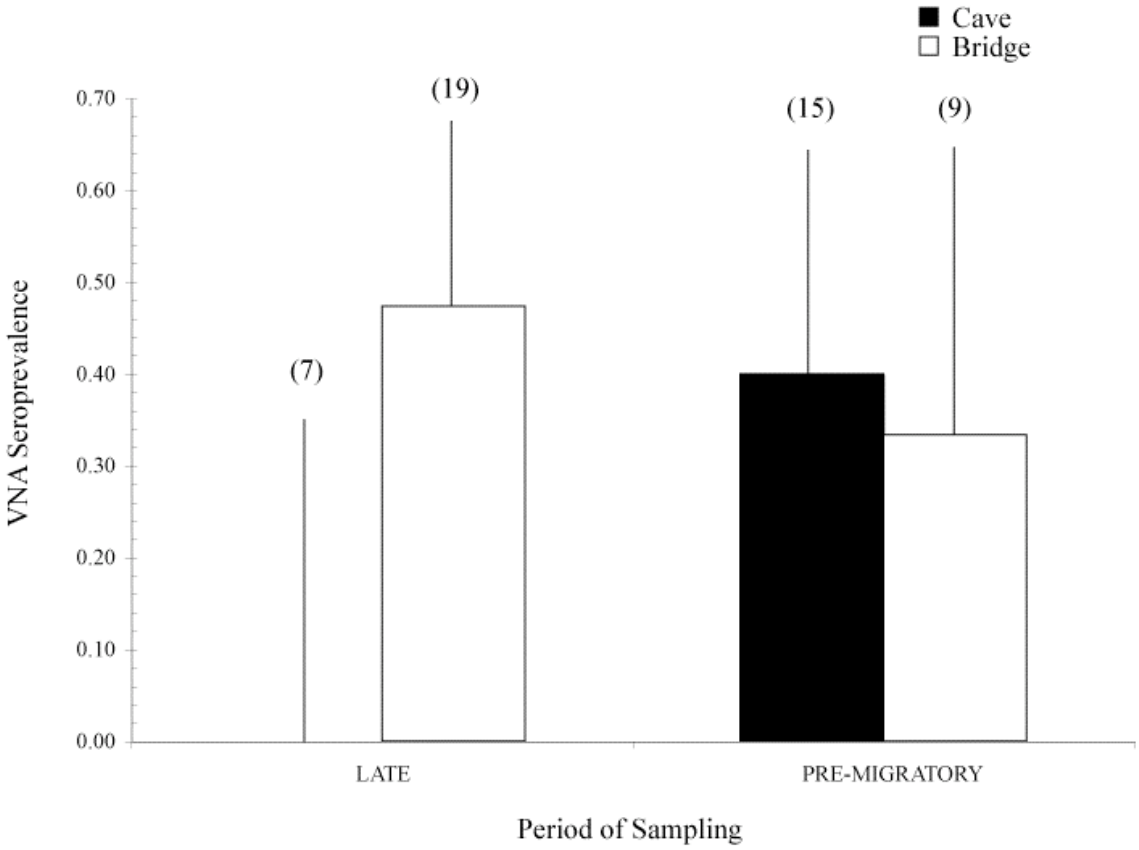

Figure 1.4 Mean VNA seroprevalence among colonies of juvenile bats, from three cave (black; DBC, JRC, FC) and three bridge colonies (white; EEB, MB, SCB), across time periods (N=50). Upper 95% confidence intervals on proportions are shown above histogram bars, and sample sizes are included parenthetically above confidence intervals.

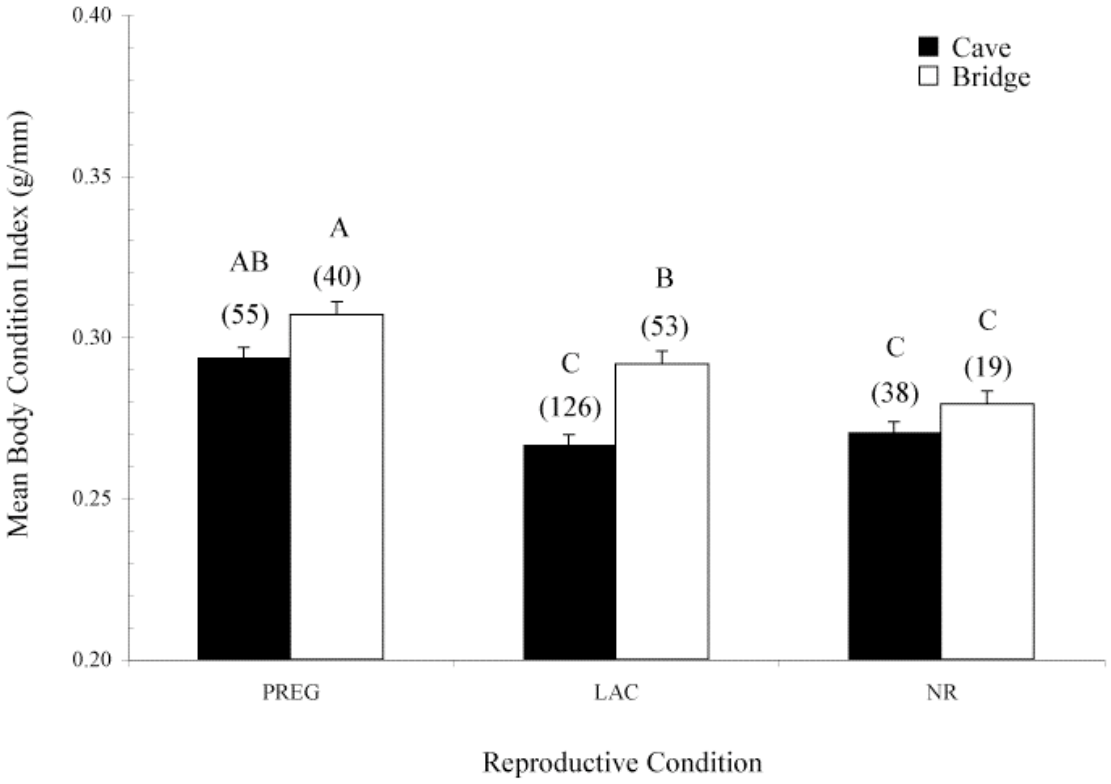

Figure 1.5 Mean (± S.E.) body condition indices of adult female bats, from three cave (black; FC, DBC, JRC) and three bridge colonies (white; MB, SCB, EEB), by reproductive status (n=331). Sample sizes are listed parenthetically above each level, and letters above bars denote significant Tukey post-hoc contrasts between levels.

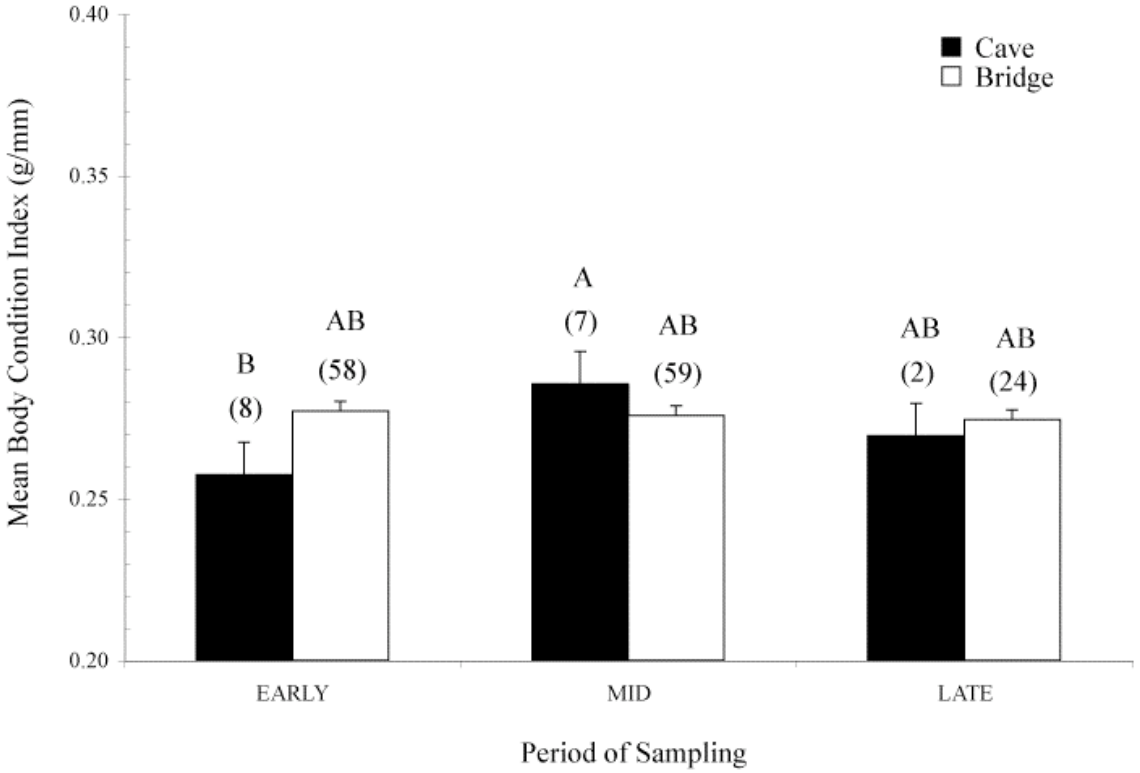

Figure 1.6 Mean (± S.E.) body condition indices of adult male bats, from one cave (black; FC), and three bridge colonies (white; MB, SCB, EEB), across time periods (n=158). Sample sizes are listed parenthetically above each level, and letters above bars denote significant Tukey post-hoc contrasts between levels.

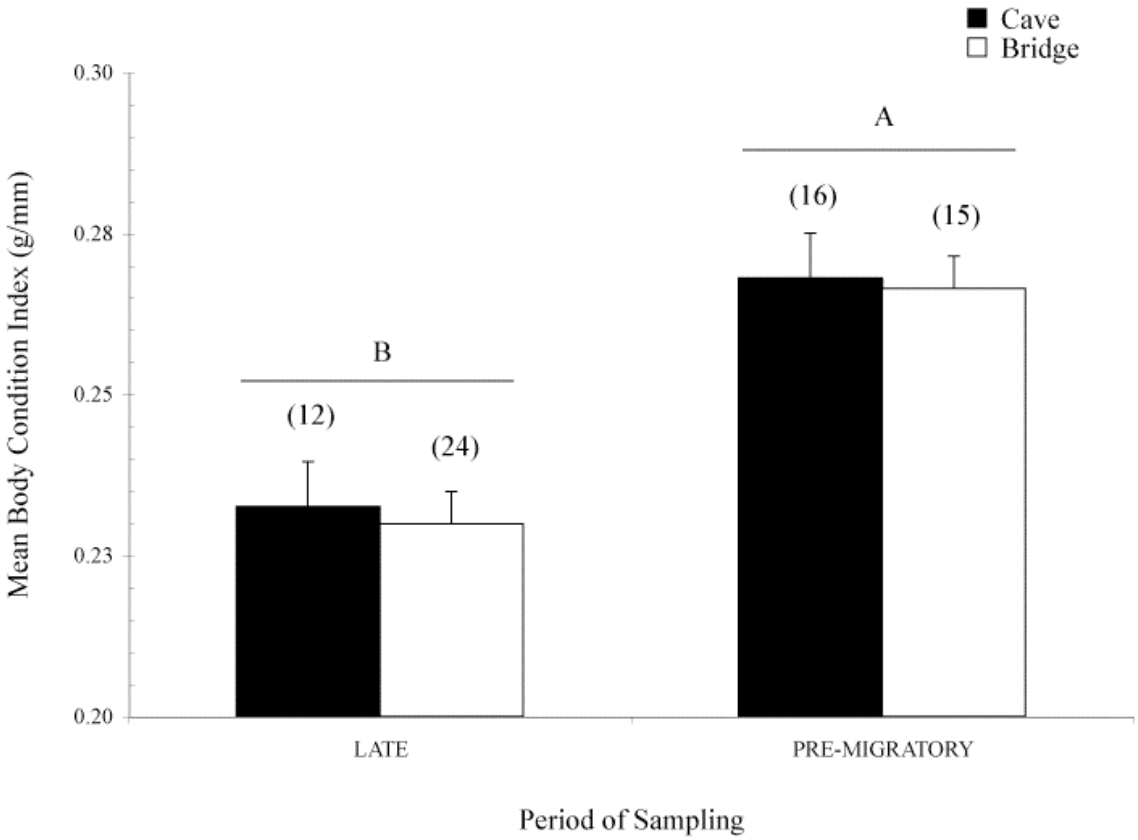

Figure 1.7 Mean (± S.E.) body condition indices of juvenile bats, from three cave (black; FC, DBC, JRC) and three bridge colonies (white; MB, SCB, EEB), across time periods (n=67). Sample sizes are listed parenthetically above each level, and letters above bars denote significant Tukey post-hoc contrasts between levels.

Appendix 2

Table 2.1 Proportion of adult Brazilian free-tailed bats with rabies VNA across six sites in the southeastern US during the Early, Mid and Late periods. Data in bold were included in the analyses.

Roost	Site	Period	Sex	N	Seroprevalence	Roost	Site	Period	Sex	N	Seroprevalence
Bridge	AUB	Early	F	0	-	*Bridge*	TCB	Early	**F**	**11**	**0.45**
			M	0	-				**M**	**5**	**0.40**
		Mid (June)	**F**	**17**	**0.35**			Mid (June)	**F**	**7**	**0.57**
			M	2	0.00				**M**	**8**	**0.13**
		Mid (July)	**F**	**18**	**0.61**			Mid (July)	**F**	**2**	**0.00**
			M	0	-				**M**	**2**	**0.50**
		Late	**F**	**15**	**0.00**			Late	**F**	**2**	**0.00**
			M	0	-				M	0	-
Bridge	BCB	Early	F	0	-	*Bat House*	GBH	Early	**F**	**11**	**0.55**
			M	**14**	**0.21**				**M**	**6**	**0.83**
		Mid (June)	**F**	**3**	**0.33**			Mid (June)	**F**	**15**	**0.60**
			M	**7**	**0.29**				**M**	**5**	**0.60**
		Mid (July)	**F**	**13**	**0.15**			Mid (July)	**F**	**9**	**0.11**
			M	**5**	**0.00**				**M**	**3**	**0.00**
		Late	**F**	**4**	**0.00**			Late	**F**	**9**	**0.33**
			M	**9**	**0.33**				**M**	**4**	**0.75**
Bridge	SACB	Early	**F**	**13**	**0.31**	*Bat House*	QBH	Early	**F**	**3**	**0.67**
			M	**6**	**0.33**				**M**	**13**	**0.69**
		Mid (June)	**F**	**9**	**0.56**			Mid (June)	**F**	**12**	**0.33**
			M	**5**	**0.80**				**M**	**5**	**0.60**
		Mid (July)	**F**	**12**	**0.33**			Mid (July)	**F**	**5**	**0.60**
			M	**8**	**0.50**				**M**	**9**	**0.44**
		Late	**F**	**1**	**0.00**			Late	**F**	**2**	**0.50**
			M	**6**	**0.00**				**M**	**1**	**1.00**

Table 2.2 Proportion of juvenile Brazilian free-tailed bats with rabies VNA across six sites in the southeastern US during the Late period.

Roost	**Site**	**Period**	**N**	**Seroprevalence**
Bridge	AUB	Late	3	0.00
Bridge	BCB	Late	5	0.00
Bridge	SACB	Late	11	0.18
Bridge	TCB	Late	16	0.19
Bat House	GBH	Late	7	0.14
Bat House	QBH	Late	3	0.33

Table 2.3 Logistic model output for VNA seroprevalence of adult Brazilian free-tailed bats from the southeastern US

Nested mixed logistic model – All Adults (n=316)

Class: Site, Roost, Period, Sex
Fixed: Roost, Period, Sex, Roost*Period, Roost*Sex, Period*Sex, Roost*Sex*Period
Random: Site [Roost]

Type 3 Effects†	df_n	df_d	$\chi 2$	F	Pr > $\chi 2$	Pr > F
Roost	1	4	15.29	15.29	0<0.0001	0.02
Period	2	302	4.18	2.09	0.12	0.12
Sex	1	302	4.08	4.08	0.04	0.04
Roost*Period	2	302	8.94	4.47	0.01	0.01
Roost*Sex	1	302	1.13	1.13	0.29	0.29
Period*Sex	2	302	4.73	1.58	0.08	0.08

Covariance parameter
Site [Roost]: estimate=0.009, s.e.=0.08

†The Roost*Period*Sex interaction term was removed from the final model, as the model did not converge with it included.

Table 2.4 Logistic model output for VNA seroprevalence of adult Brazilian free-tailed bats from bridge colonies in the southeastern US

Non-nested mixed logistic model – Bridges Adults (n=204)						
Class: Site, Period, Sex						
Fixed: Period, Sex, ln_BCI					Random: Site	
Type 3 Effects	df_n	df_d	$\chi 2$	F	Pr > $\chi 2$	Pr > F
Period	2	196	10.05	5.03	0.007	0.007
Sex	1	196	0.04	0.04	0.85	0.85
Ln_bci	1	196	2.79	2.79	0.10	0.10
Covariance parameter						
Site: estimate=0.01,	s.e.=0.09					

Non-nested mixed logistic model – Bridge Adult Females[§] (n=124)						
Class: Site, Repc						
Fixed: Repc					Random: Site	
Type 3 Effects	df_n	df_d	$\chi 2$	F	Pr > $\chi 2$	Pr > F
Repc	2	118	7.43	3.71	0.02	0.03
Covariance parameter						
Site:estimate=0.04,	s.e.=0.20					

[§]Females of undetermined reproductive status were excluded (n=3).

Non-nested mixed logistic model – Bridge Adult Females (n=127)						
Class: Site, Period						
Fixed: Period					Random: Site	
Type 3 Effects	df_n	df_d	$\chi 2$	F	Pr > $\chi 2$	Pr > F
Period	Model did not converge.				-	-
Covariance parameter						
Site:-						

Non-nested mixed logistic model – Bridge Adult Males (n=77)						
Class: Site, Period						
Fixed: Period, BCI					Random: Site	
Type 3 Effects	df_n	df_d	$\chi 2$	F	Pr > $\chi 2$	Pr > F
Period	2	70	1.43	0.71	0.49	0.49
BCI	1	70	1.35	1.35	0.25	0.25
Covariance parameter						
Site: estimate=0.15, s.e.=0.36						

Table 2.5 Logistic model output for VNA seroprevalence of adult Brazilian free-tailed bats from bat house colonies in the southeastern US

Non-nested mixed logistic model – Bat Houses Adults (n=112)						
Class: Site, Sex, Period						
Fixed: Sex, Period					Random: Site	
Type 3 Effects	df_n	df_d	$\chi 2$	F	Pr > $\chi 2$	Pr > F
Sex	1	105	3.26	3.26	0.07	0.07
Period	2	105	4.31	2.16	0.12	0.12
Sex*Period	2	105	1.78	0.89	0.41	0.41
Covariance parameter						
Site: estimate=1.6e-19						

Non-nested mixed logistic model – Bat House Adult Females (n=63)						
Class: Site, Repc						
Fixed: Repc					Random: Site	
Type 3 Effects	df_n	df_d	$\chi 2$	F	Pr > $\chi 2$	Pr > F
Repc	2	59	0.98	0.49	0.61	0.62
Covariance parameter						
Site: estimate=3.56e-22						

[§]Females of undetermined reproductive status were excluded (n=3).

Non-nested mixed logistic model – Bat House Adult Females (n=66)						
Class: Site, Period						
Fixed: Period					Random: Site	
Type 3 Effects	df_n	df_d	$\chi 2$	F	Pr > $\chi 2$	Pr > F
Period	2	62	1.32	0.66	0.52	0.52
Covariance parameter						
Site: estimate=9.53e-21						

Non-nested mixed logistic model – Bat House Adult Males (n=46)						
Class: Site, Period						
Fixed: Period					Random: Site	
Type 3 Effects	df_n	df_d	$\chi 2$	F	Pr > $\chi 2$	Pr > F
Period	2	42	4.09	2.04	0.13	0.14
Covariance parameter						
Site: estimate=4e-20						

Table 2.6 Logistic model output for VNA seroprevalence of juvenile Brazilian free-tailed bats from the southeastern US during the Late period.

Nested mixed logistic model – Juveniles – Late period (n=45)						
Class: Site, Roost						
Fixed: Roost, BCI					Random: Site [Roost]	
Type 3 Effects	df_n	df_d	$\chi 2$	F	Pr > $\chi 2$	Pr > F
Roost	1	4	0.10	0.10	0.75	0.76
BCI	1	38	2.92	2.92	0.09	0.10
Covariance parameter						
Site [Roost]: estimate=1.59e-19						

Non-nested mixed logistic model – Juveniles – Late period (n=45)						
Class: Site, Sex						
Fixed: Sex, BCI					Random: Site	
Type 3 Effects	df_n	df_d	$\chi 2$	F	Pr > $\chi 2$	Pr > F
Sex	1	37	0.61	0.61	0.44	0.44
BCI	1	37	3.42	3.42	0.06	0.07
Covariance parameter						
Site: estimate=2.69e-18						

Table 2.7 Comparisons on VNA seroprevalence between adult and juvenile cohorts during the Late period in the southeastern US.

Nested mixed logistic model – Late period (n=98)						
Class: Site, Roost, Age						
Fixed: Roost, Age, Roost*Age					Random: Site [Roost]	
Type 3 Effects	df_n	df_d	$\chi 2$	F	Pr > $\chi 2$	Pr > F
Roost	1	4	5.46	5.46	0.01	0.08
Age	1	90	0.38	0.38	0.54	0.54
Roost*Age	1	90	2.78	2.78	0.10	0.10
Covariance parameter						
Site [Roost]: estimate=1.4e-18						

Table 2.8 ANOVA model output for body condition of adult Brazilian free-tailed bats from the southeastern US.

Nested mixed ANOVA model – All Adults[§] (n=362)				
Class: Site, Roost, Sex, Period				
Fixed: Roost, Sex, Period, Roost*Sex, Period*Sex, Roost*Period, Roost*Sex*Period				
Random: Site [Roost]				
Type 3 Effects	df_n	df_d	F	Pr > F
Roost	1	350	12.13	0.0006
Period	2	350	15.22	<0.0001
Sex	1	350	76.58	<0.0001
Roost*Sex	1	350	3.44	0.06
Roost*Period	2	350	11.93	<0.0001
Sex*Period	1	350	43.53	<0.0001
Roost*Sex*Period	2	350	6.44	0.002
Covariance parameter				
Site [Roost]: estimate=0				

[§]Bats 3171, 3219, and 3260 (2 females, 1 male) were outliers, and excluded to achieve normality of model residuals.

Table 2.9 ANOVA model output by gender, for body condition of adult Brazilian free-tailed bats from the southeastern US.

Nested mixed ANOVA model – Adult Females[§] (n=213)

Class: Site, Roost, Repc
Fixed: Roost, Repc, Roost*Repc — Random: Site [Roost]

Type 3 Effects	df_n	df_d	F	Pr > F
Roost	1	5.22	3.31	0.13
Repc	2	201	20.40	<0.0001
Roost*Repc	2	201	4.87	0.009

Covariance parameter
Site [Roost]: z=0.89, p=0.19

[§]Bat 3171 was an outlier, and excluded to achieve normality of model residuals. Bats of undetermined reproductive status were also excluded (n=6).

Nested mixed ANOVA model – Adult Females[§] (n=219)

Class: Site, Roost, Period
Fixed: Roost, Period, Roost*Period — Random: Site [Roost]

Type 3 Effects	df_n	df_d	F	Pr > F
Roost	1	4.45	7.54	0.05
Period	2	197	20.40	<0.0001
Roost*Period	2	197	14.66	<0.0001

Covariance parameter
Site [Roost]: z=0.55, p=0.29

[§]Bat 3171 was an outlier, and excluded to achieve normality of model residuals.

Nested mixed ANOVA model – Adult Males (n=143)

Class: Site, Roost, Period
Fixed: Roost, Period, Roost*Period — Random: Site [Roost]

Type 3 Effects	df_n	df_d	F	Pr > F
Roost	1	3.58	0.65	0.47
Period	2	136	67.63	<0.0001
Roost*Period	2	136	0.47	0.62

Covariance parameter
Site [Roost]: z=0.75, p=0.23

Table 2.10 ANOVA model output for body condition of juvenile Brazilian free-tailed bats from the southeastern US.

Nested mixed ANOVA model - Juveniles (n=48)				
Class: Site, Sex, Roost				
Fixed: Sex, Roost, Sex*Roost				Random: Site [Roost]
Type 3 Effects	df_n	df_d	F	Pr > F
Roost	1	4.64	2.23	0.20
Sex	1	40.6	1.33	0.26
Roost*Sex	1	40.6	0.02	0.90
Covariance parameter				
Site [roost]: z=1.15, p=0.12				

Non-nested mixed ANOVA model - Juveniles (n=48)				
Class: Site, Sex				
Fixed: Sex				Random: Site
Type 3 Effects	df_n	df_d	F	Pr > F
Sex	1	41	2.80	0.10
Covariance parameter				
Site: z=1.30, p=0.10				

Table 2.11 Comparisons of body condition and body size across adult and juvenile cohorts during the Late period.

Nested mixed ANOVA model – Late period Body Condition (n=104)				
Class: Site, Roost, Age				
Fixed: Roost, Age, Roost*Age				Random: Site [Roost]
Type 3 Effects	df_n	df_d	F	Pr > F
Roost	1	4.26	3.24	0.14
Age	1	98.2	75.96	<0.0001
Roost*Age	1	98.2	0.13	0.72
Covariance parameter				
Site [roost]: z=1.22, p=0.11				

Nested mixed ANOVA model – Late period Forearm Length (n=104)				
Class: Site, Roost, Age				
Fixed: Roost, Age, Roost*Age				Random: Site [Roost]
Type 3 Effects	df_n	df_d	F	Pr > F
Roost	1	5.08	0.00	1.0
Age	1	98.5	0.35	0.56
Roost*Age	1	98.5	3.20	0.08
Covariance parameter				
Site [roost]: z=0.42, p=0.34				

Table 2.12 Model test results for VNA seroprevalence data from adult bats in Florida and Georgia.

Model	Factors	AIC	AICC	K	I
1	rt, sex, pd, rt*sex, rt*pd, sex*pd, rt*sex*pd, site (rt)	406.62	408.44	42	3.96
2	rt, sex, pd, rt*sex, rt*pd, sex*pd, rt*sex*pd, bci, site (rt)	407.62	409.67	43	4.96
3	**rt, sex, pd, rt*sex, rt*pd, sex*pd, site (rt)**	**404.35**	**405.75**	**30**	**1.69**
4	rt, sex, pd, rt*sex, rt*pd, sex*pd, bci, site (rt)	405.39	406.99	31	2.73
5	rt, sex, pd, rt*sex, rt*pd, site (rt)	407.93	408.96	24	5.27
6	rt, sex, pd, rt*sex, rt*pd, bci, site (rt)	409.61	410.81	25	6.95
7	rt, sex, pd, rt*sex, sex*pd, site (rt)	411.69	412.72	24	9.03
8	rt, sex, pd, rt*sex, sex*pd, bci, site (rt)	411.85	413.06	25	9.19
9	**rt, sex, pd, pd*sex, rt*pd, site (rt)**	**402.66**	**403.87**	**26**	**0**
10	**rt, sex, pd, pd*sex, rt*pd, bci, site (rt)**	**403.70**	**405.10**	**27**	**1.04**
11	rt, sex, pd, pd*sex, site (rt)	410.94	411.80	20	8.28
12	rt, sex, pd, pd*sex, bci, site (rt)	411.05	412.08	21	8.39
13	rt, sex, pd, pd*rt, site (rt)	406.71	407.58	20	4.05
14	rt, sex, pd, pd*rt, bci, site (rt)	408.40	409.43	21	5.74
15	rt, sex, pd, sex*rt, site (rt)	413.12	413.84	18	10.46
16	rt, sex, pd, sex*rt, bci, site (rt)	414.25	415.12	19	11.59
17	rt, sex, pd, site (rt)	412.52	413.11	14	9.86
18	rt, sex, pd, bci, site (rt)	413.62	414.34	15	10.96
19	rt, pd, rt*pd, site (rt)	405.51	406.23	18	2.85
20	rt, pd, rt*pd, bci, site (rt)	407.45	408.32	19	4.79
21	rt, pd, site (rt)	411.46	411.93	12	8.8
22	rt, pd, bci, site (rt)	413.10	413.69	13	10.44
23	rt, sex, rt*sex, site (rt)	416.68	417.15	15	14.02
24	rt, sex, rt*sex, bci, site (rt)	418.60	419.19	16	15.94
25	rt, sex, site (rt)	416.56	416.92	11	13.9
26	rt, sex, bci, site (rt)	418.50	418.97	12	15.84
27	pd, sex, pd*sex, site	410.94	411.80	18	8.28
28	pd, sex, pd*sex, bci, site	411.05	412.08	19	8.39
29	pd, sex, site	412.52	413.11	12	9.86
30	pd, sex, bci, site	413.62	414.34	13	10.96
31	rt, site (rt)	415.50	415.78	9	12.84
32	rt, bci, site (rt)	417.50	417.86	10	14.84
33	sex, site	416.56	416.92	9	13.90
34	sex, bci, site	418.50	418.97	10	15.84
35	pd, site	411.46	411.93	10	8.80
36	pd, bci, site	413.10	413.69	11	10.44
37	site	415.50	415.78	7	12.84
38	site, bci	417.50	417.86	8	14.84

Table 2.13 Model test results for VNA seroprevalence data from juvenile bats in Florida and Georgia.

Model	Factors	AICC	K	I
1	rt, sex, rt*sex, site (rt)	52.91	15	5.35
2	rt, sex, site (rt)	52.27	11	4.71
3	rt, sex, bci, site (rt)	49.97	12	2.41
4	rt, site (rt)	49.64	9	2.08
5	**rt, bci, site (rt)**	**47.56**	**10**	**0**
6	sex, site	52.27	9	4.71
7	sex, bci, site	49.97	10	2.41
8	site	49.64	7	2.08
9	**site, bci**	**47.56**	**8**	**0**

Table 2.14 Model test results for body condition data from adult bats in Florida and Georgia.

Model	Factors	AIC	AICC	K	I
1	**rt, sex, pd, rt*sex, rt*pd, sex*pd, rt*sex*pd**	**-819.4**	**-819.4**	**36**	**1.6**
2	rt, sex, pd, rt*sex, rt*pd, sex*pd	-815.7	-815.7	24	5.3
3	rt, sex, pd, rt*sex, rt*pd	-758.9	-758.9	18	62.1
4	rt, sex, pd, rt*sex, sex*pd	-800.5	-800.4	18	20.5
5	**rt, sex, pd, pd*sex, rt*pd**	**-821.0**	**-821.0**	**20**	**0.0**
6	rt, sex, pd, pd*sex	-806.7	-806.7	14	14.3
7	rt, sex, pd, pd*rt	-763.0	-763.0	14	58.0
8	rt, sex, pd, sex*rt	-754.3	-754.2	12	66.7
9	rt, sex, pd	-759.9	-759.8	8	61.1
10	rt, pd, rt*pd	-733.5	-733.5	12	87.5
11	rt, pd	-733.9	-733.8	6	87.1
12	rt, sex, rt*sex	-735.6	-735.6	9	85.4
13	rt, sex	-740.0	-739.9	5	81.0
14	pd, sex, pd*sex	-811.6	-811.6	12	9.4
15	pd, sex	-765.6	-765.6	6	55.4
16	rt	-711.0	-710.9	3	110.0
17	sex	-746.4	-746.4	3	74.6
18	pd	-739.6	-739.6	4	81.4

Table 2.15 Model test results for body condition data from juvenile bats in Florida and Georgia.

Model	Factors	AICC	K	I
1	rt, sex, rt*sex	-230.7	9	13.2
2	rt, sex	-237.6	5	6.3
3	**Rt**	**-243.9**	**3**	**0.0**
4	**Sex**	**-242.4**	**3**	**1.5**

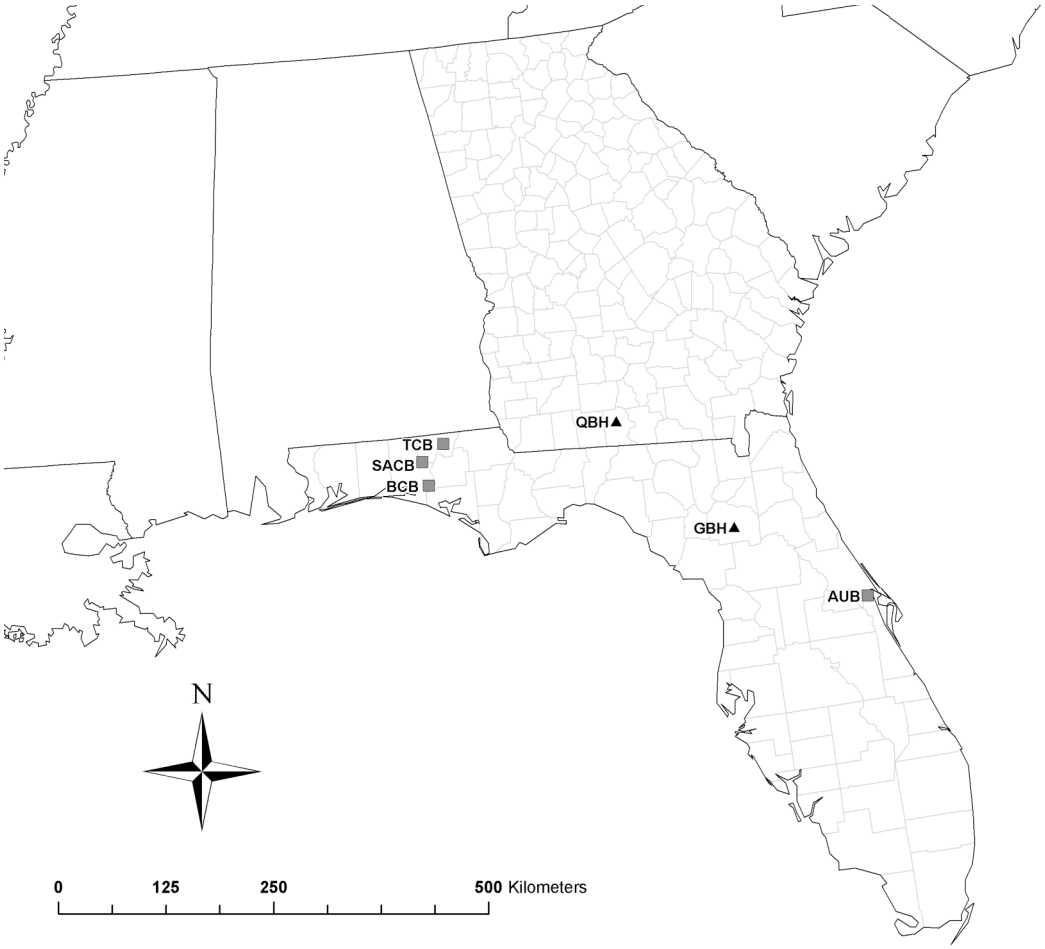

Figure 2.1 The geographic location of all sites sampled in the southeastern US. Bridge colonies (gray squares) include: Aurantia Bridge (AUB), Black Creek Bridge (BCB), Sandy Creek Bridge (SACB), and Tenmile Creek Bridge (TCB). Bat house colonies (black triangles) include: Quitman Bat Houses (QBH) and Gainesville Bat House (GBH).

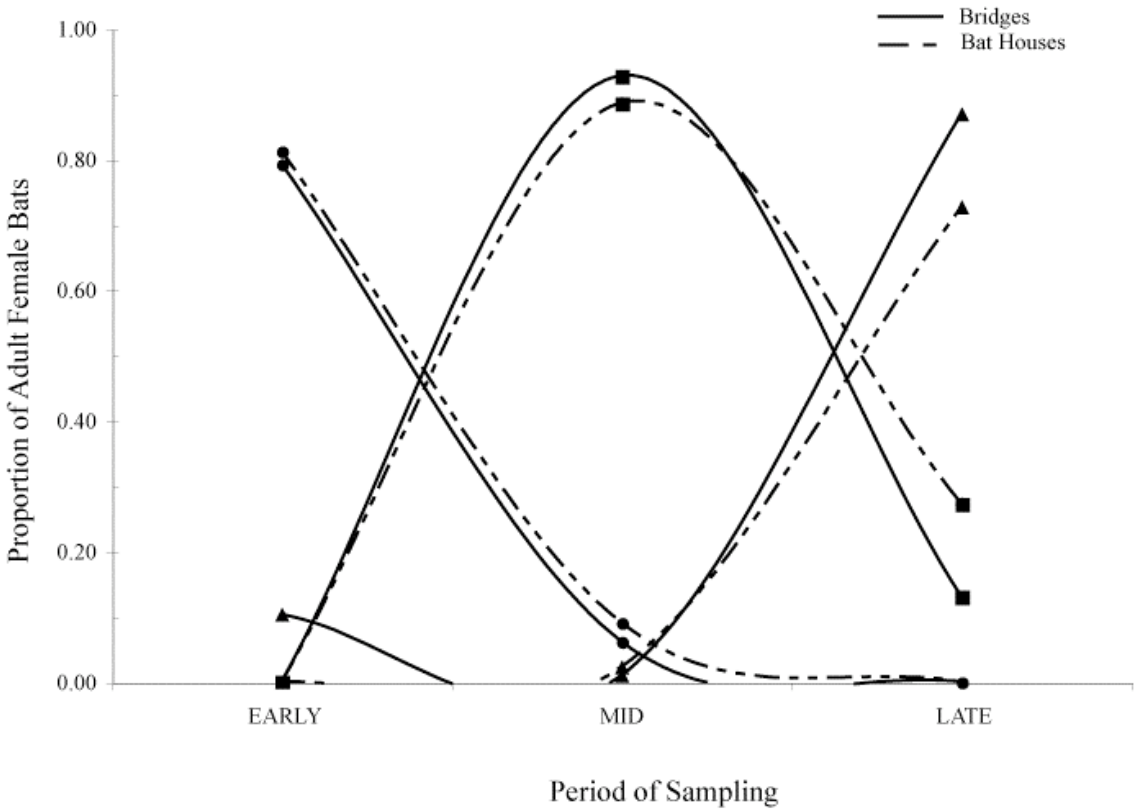

Figure 2.2 The reproductive activity of adult female bats measured during Early, Mid, and Late periods. Solid lines bridge colonies, and dashed lines represent bat house colonies. Reproductive status was: pregnant (circle), lactating (square), or non-reproductive (triangle). Females of undetermined reproductive status are not shown (n=6).

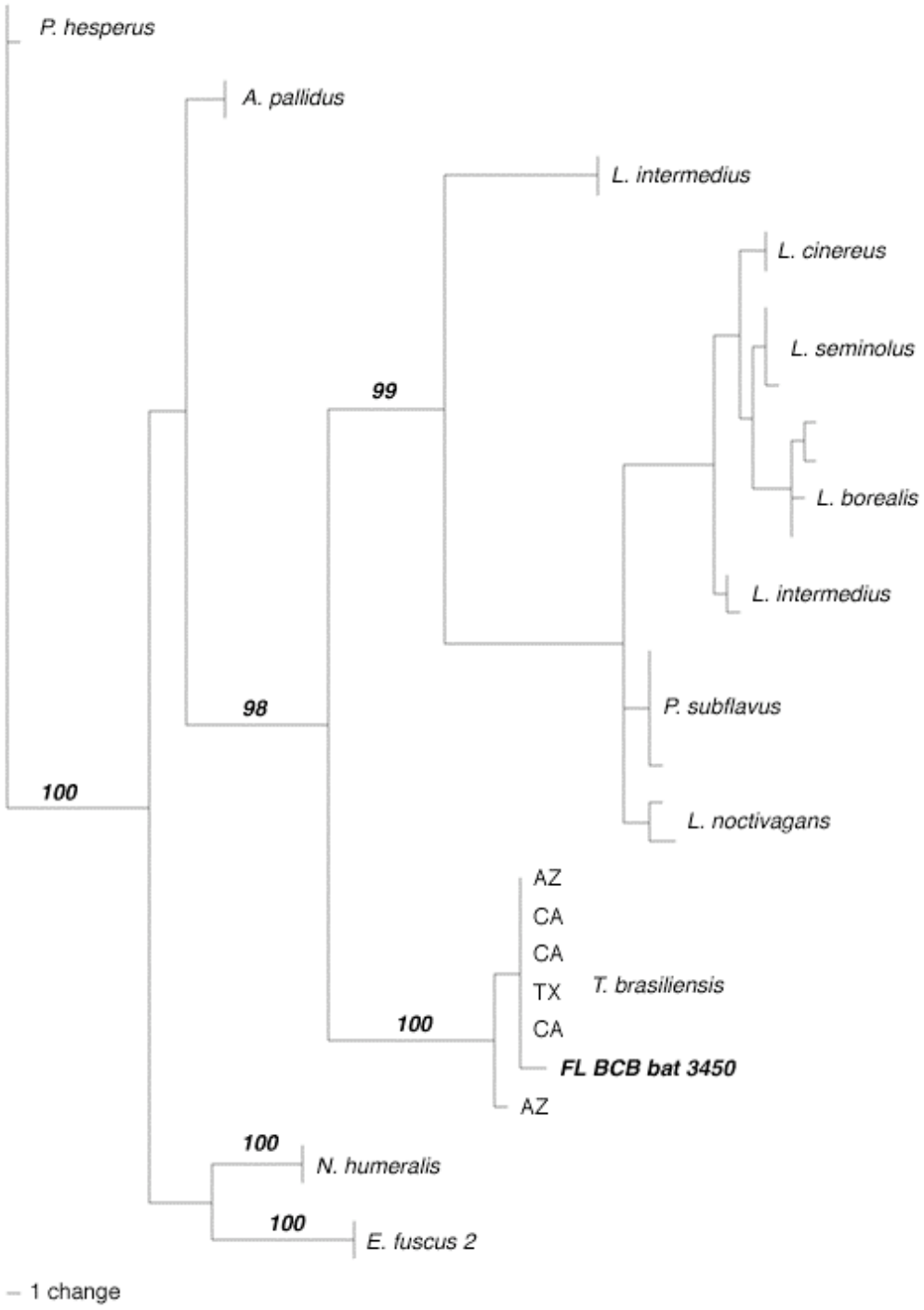

Figure 2.3 Phylogeny of rabies virus nucleoprotein sequence variants from insectivorous bats in the United States (sequences from D. Streicker). State abbreviations are provided for *T. brasiliensis* variants, and the positive swab from Black Creek Bridge (BCB, FL) is italicized and highlighted in bold. Bayesian posterior probabilities are listed above selected nodes.

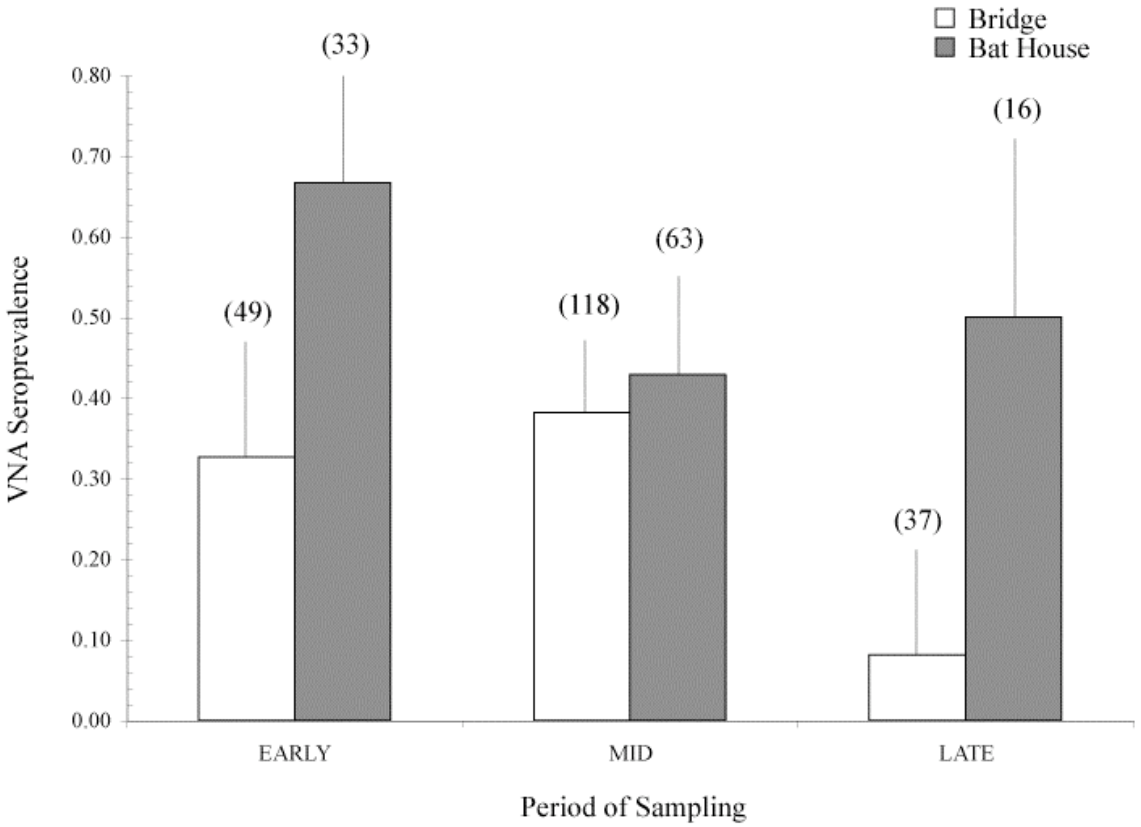

Figure 2.4 Mean VNA seroprevalence among adult bats, from four bridge (white; AUB, BCB, SACB, TCB) and two bat house (gray; QBH, GBH) colonies, across time periods (n=316). Upper 95% confidence intervals on proportions are shown above histogram bars, and sample sizes are included parenthetically above confidence intervals.

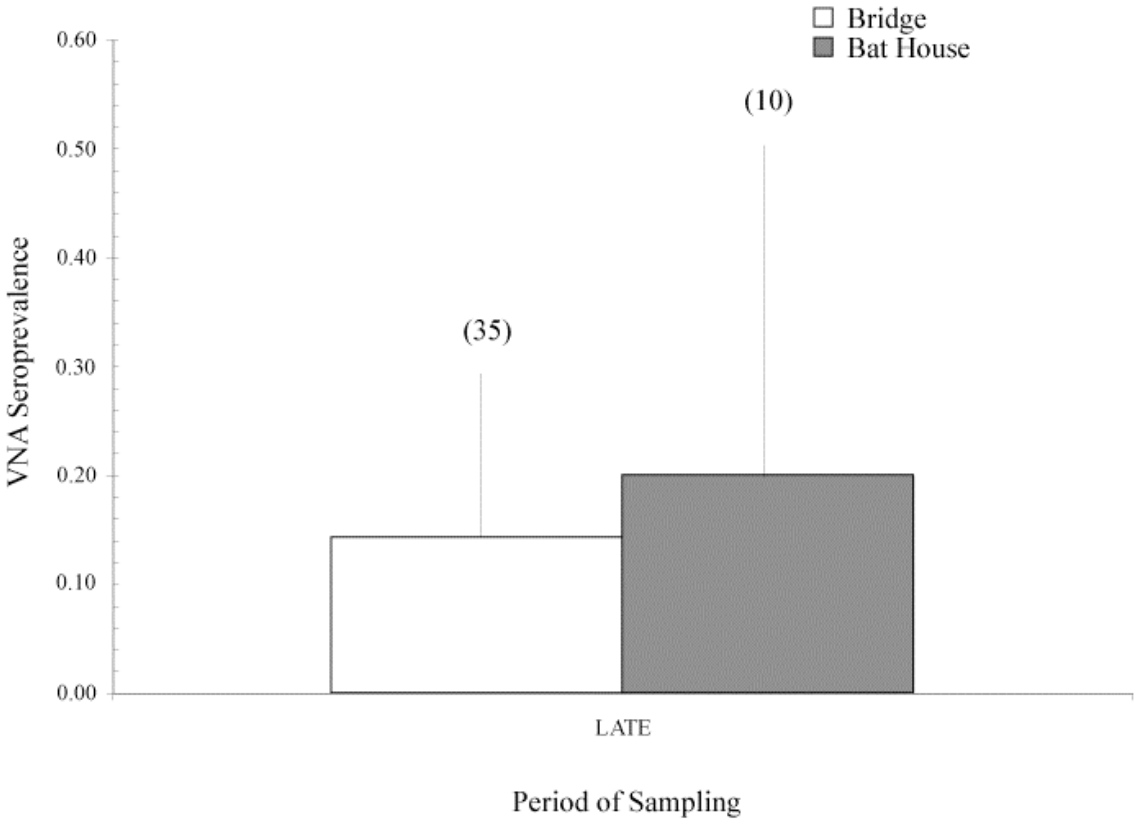

Figure 2.5 Mean VNA seroprevalence among juvenile bats, from four bridge (white; AUB, BCB, SACB, TCB) and two bat house (gray; QBH, GBH) colonies, across time periods (n=45). Upper 95% confidence intervals on proportions are shown above histogram bars, and sample sizes are included parenthetically above confidence intervals.

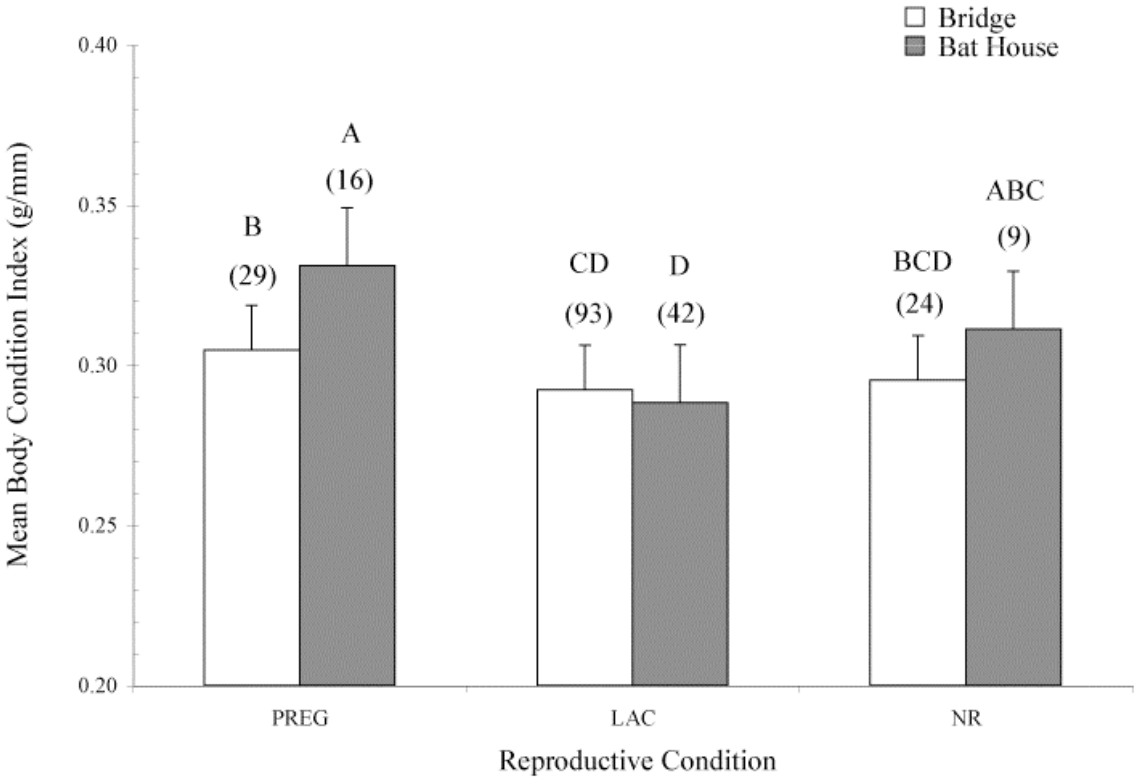

Figure 2.6 Mean (± S.E.) body condition indices among adult female bats, summarized across four bridge (white; AUB, BCB, SACB, TCB) and two bat house (gray; QBH, GBH) colonies, by reproductive status (n=213). Sample sizes are listed parenthetically above each level, and letters above bars denote significant Tukey post-hoc contrasts between levels.

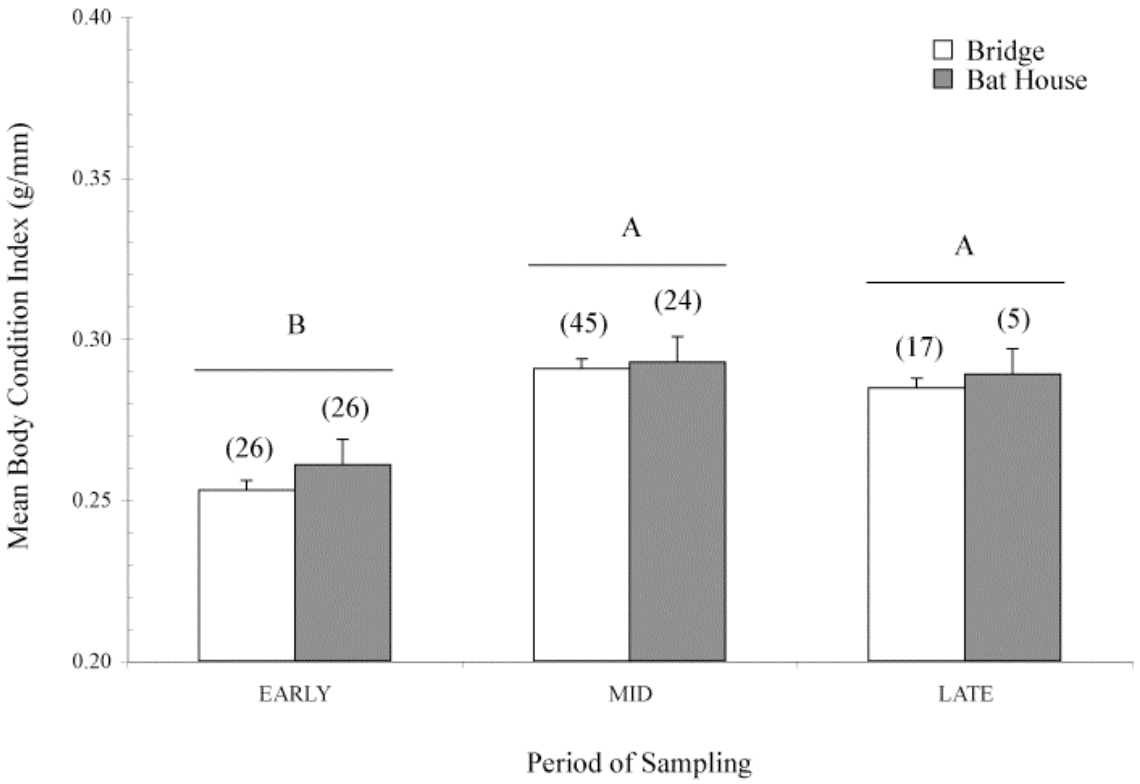

Figure 2.7 Mean (± S.E.) body condition indices among adult male bats, summarized across three bridge (white; BCB, SACB, TCB) and two bat house (gray; QBH, GBH) colonies, across time periods (n=143). Sample sizes are listed parenthetically above each level, and letters above bars denote significant Tukey post-hoc contrasts between levels.

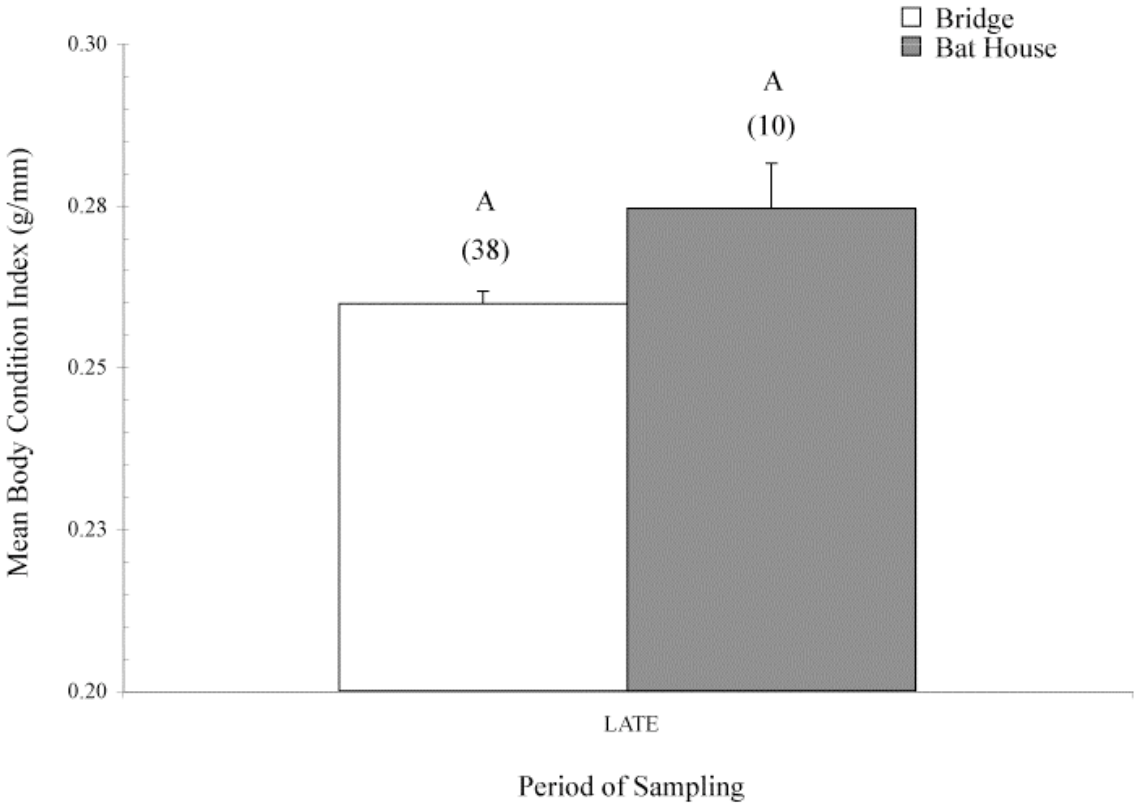

Figure 2.8 Mean (± S.E.) body condition indices among juvenile bats, summarized across four bridge (white; AUB, BCB, SACB, TCB) and two bat house (gray; QBH, GBH) colonies, during the Late period (n=48). Sample sizes are listed parenthetically above each level, and letters above bars denote significant Tukey post-hoc contrasts between levels.

Appendix 3

Table 3.1 Logistic model output for VNA seroprevalence of adult Brazilian free-tailed bats in the southern US.

Nested mixed logistic model – Roost Type – All Adults (n=779)

Class: Site, Roost, Sex, Period
Fixed: Roost, Sex, Period, Roost*Sex, Roost*Period, Sex*Period, Roost*Sex*Period
Random: Site [Roost]

Type 3 Effects†	df_n	df_d	χ_2	F	P>χ_2	P>F
Roost	2	9	2.76	1.38	0.25	0.30
Sex	1	756	1.64	1.64	0.20	0.20
Period	2	756	1.98	0.99	0.37	0.37
Roost*Sex	2	756	2.42	1.21	0.30	0.30
Roost*Period	2	756	13.25	3.31	0.01	0.01
Period*Sex	2	756	1.05	0.53	0.59	0.59

Covariance parameter
Site [Roost]: estimate=0.26, s.e.=0.16

†The Roost*Sex*Period interaction term (p=0.66) was removed from the final model.

Nested mixed logistic model – Regional – Bridge Adults (n=435)

Class: Site, Region, Sex, Period
Fixed: Region, Sex, Period, Region*Sex, Region*Period, Sex*Period, Region*Sex*Period
Random: Site [Region]

Type 3 Effects†	df_n	df_d	χ_2	F	P>χ_2	P>F
Region	1	5	9.30	9.30	0.002	0.03
Sex	1	420	0.20	0.20	0.65	0.66
Period	2	420	8.35	4.17	0.02	0.02
Region*Sex	1	420	0.00	0.00	1.0	1.0
Region*Period	2	420	6.61	3.30	0.04	0.04
Period*Sex	2	420	0.57	0.28	0.75	0.75

Covariance parameter
Site [Region]: estimate=0.09, s.e.=0.10

†The Region*Sex*Period interaction term was removed from the final model, as the model did not converge with it included.

Table 3.2 Logistic model output for VNA seroprevalence of juvenile Brazilian free-tailed bats in the southern US.

Nested mixed logistic model – Roost Type – Juveniles, Late Period (n=64)						
Class: Site, Roost						
Fixed: Roost						Random: Site [Roost]
Type 3 Effects	df_n	df_d	χ_2	F	$P>\chi_2$	P>F
Roost	1	6	0.05	0.05	0.83	0.83
Covariance parameter						
Site [Roost]: estimate=0.33, s.e.=0.56						

Nested mixed logistic model – Regional – Juveniles, Late Period (n=54)						
Class: Site, Region						
Fixed: Region						Random: Site [Region]
Type 3 Effects	df_n	df_d	χ_2	F	$P>\chi_2$	P>F
Region	1	4	6.40	6.40	0.01	0.06
Covariance parameter						
Site [Region]: estimate=8.67e-19						

Non-nested mixed logistic model – Juveniles, Late Period (n=71)						
Class: Site, Sex						
Fixed: Sex						Random: Site
Type 3 Effects	df_n	df_d	χ_2	F	$P>\chi_2$	P>F
Sex	1	59	2.50	2.50	0.11	0.12
Covariance parameter						
Site: estimate=0.24, s.e.=0.43						

Table 3.3 ANOVA model output for body condition of adult female Brazilian free-tailed bats from colonies in the southern US.

Nested mixed ANOVA model – Roost Type – Adult Females[§] (n=545)

Class: Site, Roost, Repc
Fixed: Roost, Repc, Roost*Repc Random: Site [Roost]

Type 3 Effects	df_n	df_d	F	P > F
Roost	2	11.1	17.6	0.0004
Repc	2	535	78.6	<0.0001
Repc*Roost	4	532	5.30	0.0003

Covariance parameter
Site (roost) z=1.43, p=0.08

[§]Females of undetermined reproductive status were excluded (n=26).

Nested mixed ANOVA model – Regional – Bridge Adult Females[§] (n=258)

Class: Site, Region, Repc
Fixed: Region, Repc, Region*Repc Random: Site [Region]

Type 3 Effects	df_n	df_d	F	P > F
Region	1	6.06	0.4	0.55
Repc	2	224	19.8	<0.0001
Region*Repc	2	224	3.23	0.04

Covariance parameter
Site [Region] z=0.91, p=0.18

[§]Females of undetermined reproductive status were excluded (n=6).

Table 3.4 ANOVA model output for body condition of adult male Brazilian free-tailed bats from colonies in the southern US.

Nested mixed ANOVA model – Roost Type – Adult Males (n=301)				
Class: Site, Roost, Period				
Fixed: Roost, Period, Roost*Period				Random: Site [Roost]
Type 3 Effects	df_n	df_d	F	P > F
Roost	2	17.2	1.02	0.38
Period	2	289	21.01	<0.0001
Roost*Period	4	290	2.17	0.07
Covariance parameter				
Site [Roost] z=0.73, p=0.23				

Nested mixed ANOVA model – Regional – Bridge Adult Males (n=229)				
Class: Site, Region, Period				
Fixed: Region, Period, Region*Period				Random: Site [Region]
Type 3 Effects	df_n	df_d	F	P>F
Region	1	4.27	0.16	0.71
Period	2	222	27.99	<0.0001
Region*Period	2	222	21.49	<0.0001
Covariance parameter				
Site [Region] z=0.96, p=0.17				

Table 3.5 ANOVA model output for body condition of juvenile Brazilian free-tailed bats from colonies in the southern US.

Nested mixed ANOVA model – Roost – Juveniles, Late Period (n=84)

Class: Site, Roost
Fixed: Roost Random: Site [Roost]

Type 3 Effects	df_n	df_d	F	P>F
Roost	2	9	2.79	0.11

Covariance parameter
Site [Roost] z=1.79, p=0.04

Non-nested mixed ANOVA model – Juveniles, Late Period (n=84)

Class: Site, Sex
Fixed: Sex Random: Site

Type 3 Effects	df_n	df_d	F	P>F
Sex	1	71	4.20	0.04

Covariance parameter
Site z=2.03, p=0.02

Nested mixed ANOVA model – Region – All Juveniles, Late Period (n=84)

Class: Site, Region
Fixed: Region Random: Site [Region]

Type 3 Effects	df_n	df_d	F	P>F
Region	1	10	12.01	0.006

Covariance parameter
Site [Region] z=1.63, p=0.05

Nested mixed ANOVA model – Region – Bridge Juveniles, Late Period (n=62)

Class: Site, Region
Fixed: Region Random: Site [Region]

Type 3 Effects	df_n	df_d	F	P>F
Region	1	5	7.56	0.04

Covariance parameter
Site [Region] z=1.25, p=0.11

Table 3.6 Model test results for VNA seroprevalence data from adult bats in the southern US.

Model	Factors	AIC	AICC	K	I
1	rt, sex, pd, rt*sex, rt*pd, sex*pd, rt*sex*pd, site (rt)	1021.88	1023.89	60	11.93
2	rt, sex, pd, rt*sex, rt*pd, sex*pd, rt*sex*pd, bci, site (rt)	1023.62	1025.79	61	13.67
3	rt, sex, pd, rt*sex, rt*pd, sex*pd, site (rt)	1016.29	1017.75	42	6.34
4	rt, sex, pd, rt*sex, rt*pd, sex*pd, bci, site (rt)	1017.91	1019.5	43	7.96
5	rt, sex, pd, rt*sex, rt*pd, site (rt)	1013.54	1014.77	36	3.59
6	rt, sex, pd, rt*sex, rt*pd, bci, site (rt)	1015.4	1016.74	37	5.45
7	rt, sex, pd, rt*sex, sex*pd, site (rt)	1022.17	1023.17	33	12.22
8	rt, sex, pd, rt*sex, sex*pd, bci, site (rt)	1023.78	1024.89	34	13.83
9	rt, sex, pd, pd*sex, rt*pd, site (rt)	1014.94	1016.17	36	4.99
10	rt, sex, pd, pd*sex, rt*pd, bci, site (rt)	1016.66	1018.00	37	6.71
11	rt, sex, pd, pd*sex, site (rt)	1022.17	1022.97	27	12.22
12	rt, sex, pd, pd*sex, bci, site (rt)	1023.77	1024.67	28	13.82
13	**rt, sex, pd, pd*rt, site (rt)**	**1011.93**	**1012.93**	**30**	**1.98**
14	rt, sex, pd, pd*rt, bci, site (rt)	1013.82	1014.93	31	3.87
15	rt, sex, pd, sex*rt, site (rt)	1018.81	1019.62	27	8.86
16	rt, sex, pd, sex*rt, bci, site (rt)	1020.42	1021.32	28	10.47
17	rt, sex, pd, site (rt)	1018.67	1019.3	21	8.72
18	rt, sex, pd, bci, site (rt)	1020.28	1020.99	22	10.33
19	**rt, pd, rt*pd, site (rt)**	**1009.95**	**1010.86**	**28**	**0**
20	**rt, pd, rt*pd, bci, site (rt)**	**1011.82**	**1012.82**	**29**	**1.87**
21	rt, pd, site (rt)	1016.78	1017.33	19	6.83
22	rt, pd, bci, site (rt)	1018.29	1018.92	20	8.34
23	rt, sex, rt*sex, site (rt)	1024.58	1025.21	24	14.63
24	rt, sex, rt*sex, bci, site (rt)	1026.03	1026.74	25	16.08
25	rt, sex, site (rt)	1023.64	1024.11	18	13.69
26	rt, sex, bci, site (rt)	1025.1	1025.65	19	15.15
27	pd, sex, pd*sex, site	1022.17	1022.97	24	12.22
28	pd, sex, pd*sex, bci, site	1023.77	1024.67	25	13.82
29	pd, sex, site	1018.67	1019.3	18	8.72
30	pd, sex, bci, site	1020.28	1020.99	19	10.33
31	rt, site (rt)	1021.76	1022.17	16	11.81
32	rt, bci, site (rt)	1023.11	1023.58	17	13.16
33	sex, site	1023.64	1024.11	15	13.69
34	sex, bci, site	1025.1	1025.65	16	15.15
35	pd, site	1016.78	1017.33	16	6.83
36	pd, bci, site	1018.29	1018.92	17	8.34
37	site	1021.76	1022.17	13	11.81
38	site, bci	1023.11	1023.58	14	13.16

Table 3.7 Model test results for VNA seroprevalence data from adult bats in bridge roosts in the southern US.

Model	Factors	AIC	AICC	K	I
1	**rg, sex, pd, rg*sex, rg*pd, sex*pd, rg*sex*pd, site (rg)**	**568.4**	**569.87**	**43**	**1.37**
2	rg, sex, pd, rg*sex, rg*pd, sex*pd, rg*sex*pd, bci, site (rg)	569.68	571.33	44	2.65
3	rg, sex, pd, rg*sex, rg*pd, sex*pd, site (rg)	573.92	575.07	31	6.89
4	rg, sex, pd, rg*sex, rg*pd, sex*pd, bci, site (rg)	574.87	576.18	32	7.84
5	rg, sex, pd, rg*sex, rg*pd, site (rg)	570.65	571.52	25	3.62
6	rg, sex, pd, rg*sex, rg*pd, bci, site (rg)	572.03	573.03	26	5
7	rg, sex, pd, rg*sex, sex*pd, site (rg)	577.76	578.62	25	10.73
8	rg, sex, pd, rg*sex, sex*pd, bci, site (rg)	577.57	578.57	26	10.54
9	rg, sex, pd, pd*sex, rg*pd, site (rg)	572.09	573.1	27	5.06
10	rg, sex, pd, pd*sex, rg*pd, bci, site (rg)	573.11	574.26	28	6.08
11	rg, sex, pd, pd*sex, site (rg)	576.02	576.77	21	8.99
12	rg, sex, pd, pd*sex, bci, site (rg)	575.94	576.81	22	8.91
13	**rg, sex, pd, pd*rg, site (rg)**	**568.87**	**569.61**	**21**	**1.84**
14	rg, sex, pd, pd*rg, bci, site (rg)	570.3	571.17	22	3.27
15	rg, sex, pd, sex*rg, site (rg)	574.31	574.94	19	7.28
16	rg, sex, pd, sex*rg, bci, site (rg)	574.65	575.39	20	7.62
17	rg, sex, pd, site (rg)	572.62	573.14	15	5.59
18	rg, sex, pd, bci, site (rg)	573.06	573.69	16	6.03
19	**rg, pd, rg*pd, site (rg)**	**567.03**	**567.66**	**19**	**0**
20	**rg, pd, rg*pd, bci, site (rg)**	**568.77**	**569.51**	**20**	**1.74**
21	rg, pd, site (rg)	571.18	571.6	13	4.15
22	rg, pd, bci, site (rg)	572.42	572.94	14	5.39
23	rg, sex, rg*sex, site (rg)	577.34	577.76	16	10.31
24	rg, sex, rg*sex, bci, site (rg)	578.27	578.79	17	11.24
25	rg, sex, site (rg)	575.4	575.74	12	8.37
26	rg, sex, bci, site (rg)	576.36	576.79	13	9.33
27	pd, sex, pd*sex, site	576.02	576.77	19	8.99
28	pd, sex, pd*sex, bci, site	575.94	576.81	20	8.91
29	pd, sex, site	572.62	573.14	13	5.59
30	pd, sex, bci, site	573.06	573.69	14	6.03
31	rg, site (rg)	573.75	574.01	10	6.72
32	rg, bci, site (rg)	575.25	575.58	11	8.22
33	sex, site	575.4	575.74	10	8.37
34	sex, bci, site	576.36	576.79	11	9.33
35	pd, site	571.18	571.6	11	4.15
36	pd, bci, site	572.42	572.94	12	5.39
37	site	573.75	574.01	8	6.72
38	site, bci	575.25	575.58	9	8.22

Table 3.8 Model test results for VNA seroprevalence data from juvenile bats in the southern US.

Model	Factors	AICC	K	I
1	**rt, sex, rt*sex, site (rt)**	**81.98**	**17**	**1.65**
2	rt, sex, rt*sex, bci, site (rt)	84.36	18	4.03
3	**rt, sex, site (rt)**	**81.76**	**13**	**1.43**
4	rt, sex, bci, site (rt)	83.97	14	3.64
5	**rt, site (rt)**	**80.33**	**11**	**0**
6	rt, bci, site (rt)	82.76	12	2.43
7	**sex, site**	**81.76**	**11**	**1.43**
8	sex, bci, site	83.97	12	3.64
9	**site**	**80.33**	**9**	**0**
10	site, bci	82.76	10	2.43

Table 3.9 Model test results for VNA seroprevalence data from juvenile bats in bridge roosts in the southern US.

Model	Factors	AICC	K	I
1	rg, sex, rg*sex, site (rg)	68.85	15	2.92
2	rg, sex, rg*sex, bci, site (rg)	71.71	16	5.78
3	**rg, sex, site (rg)**	**66.09**	**11**	**0.16**
4	rg, sex, bci, site (rg)	68.83	12	2.9
5	**rg, site (rg)**	**65.93**	**9**	**0**
6	rg, bci, site (rg)	68.37	10	2.44
7	**sex, site**	**66.09**	**9**	**0.16**
8	sex, bci, site	68.83	10	2.9
9	**site**	**65.93**	**7**	**0**
10	site, bci	68.37	8	2.44

Table 3.10 Model test results for body condition data from adult bats in the southern US*.

Model	Factors	AIC	AICC	K	I
1	**rt, sex, pd, rt*sex, rt*pd, sex*pd, rt*sex*pd**	**-1894.3**	**-1894.3**	**48**	**0**
2	rt, sex, pd, rt*sex, rt*pd, sex*pd	-1882.1	-1882.1	30	12.2
3	rt, sex, pd, rt*sex, rt*pd	-1818.6	-1818.6	24	75.7
4	rt, sex, pd, rt*sex, sex*pd	-1885.3	-1885.3	21	9
5	rt, sex, pd, pd*sex, rt*pd	-1884.1	-1884.1	24	10.2
6	rt, sex, pd, pd*sex	-1883.8	-1883.8	15	10.5
7	rt, sex, pd, pd*rt	-1829.4	-1829.4	18	64.9
8	rt, sex, pd, sex*rt	-1790.1	-1790.1	15	104.2
9	rt, sex, pd	-1797.6	-1797.6	9	96.7
10	rt, pd, rt*pd	-1739.7	-1739.7	16	154.6
11	rt, pd	-1715.9	-1715.9	7	178.4
12	rt, sex, rt*sex	-1803.4	-1803.4	12	90.9
13	rt, sex	-1810.8	-1810.8	6	83.5
14	pd, sex, pd*sex	-1875.7	-1875.7	12	18.6
15	pd, sex	-1789.3	-1789.3	6	105
16	rt	-1729.8	-1729.8	4	91.4
17	sex	-1802.9	-1802.9	3	91.4
18	pd	-1719.6	-1719.5	4	174.7

*bats 1133, 3171, 1245, 1293, 3219 were removed to achieve normally distributed residuals to the model

Table 3.11 Model test results for body condition data from adult bats in bridge roosts in the southern US*.

Model	Factors	AIC	AICC	K	I
1	rg, sex, pd, rg*sex, rg*pd, sex*pd, rg*sex*pd	-1106.2	-1106.2	36	10
2	rg, sex, pd, rg*sex, rg*pd, sex*pd	-1112.2	-1112.2	24	4
3	rg, sex, pd, rg*sex, rg*pd	-1097.6	-1097.6	18	18.6
4	rg, sex, pd, rg*sex, sex*pd	-1067.8	-1067.8	18	48.4
5	**rg, sex, pd, pd*sex, rg*pd**	**-1116.2**	**-1116.1**	**20**	**0.0**
6	rg, sex, pd, pd*sex	-1073.0	-1073.0	14	43.2
7	rg, sex, pd, pd*rg	-1099.5	-1099.5	14	16.7
8	rg, sex, pd, sex*rg	-1060.6	-1060.6	12	55.6
9	rg, sex, pd	-1064.5	-1064.5	8	51.7
10	rg, pd, rg*pd	-1023.2	-1023.2	12	93.0
11	rg, pd	-998.10	-998.0	6	118.1
12	rg, sex, rg*sex	-1067.3	-1067.3	9	48.9
13	rg, sex	-1071.9	-1071.9	5	44.3
14	pd, sex, pd*sex	-1080.4	-1080.4	12	35.8
15	pd, sex	-1071.7	-1071.7	6	44.5
16	rg	-1004.9	-1004.9	3	111.3
17	sex	-1079.1	-1079.1	3	37.1
18	pd	-1003.9	-1003.8	4	112.3

*bat 1133 was removed to achieve normally distributed residuals to the model

Table 3.12 Model test results for body condition data from juvenile bats in the southern US.

Model	Factors	AICC	K	I
1	rt, sex, rt*sex	-379.0	12	21.8
2	rt, sex	-392.5	6	8.3
3	rt	-397.5	4	3.3
4	**sex**	**-400.8**	**3**	**0.0**

Table 3.13 Model test results for body condition data from juvenile bats in bridge roosts in the southern US.

Model	Factors	AICC	K	I
1	rg, sex, rg*sex	-294.9	9	13.2
2	rg, sex	-302.4	5	5.7
3	**rg**	**-308.1**	**3**	**0.0**
4	sex	-304.8	3	3.3

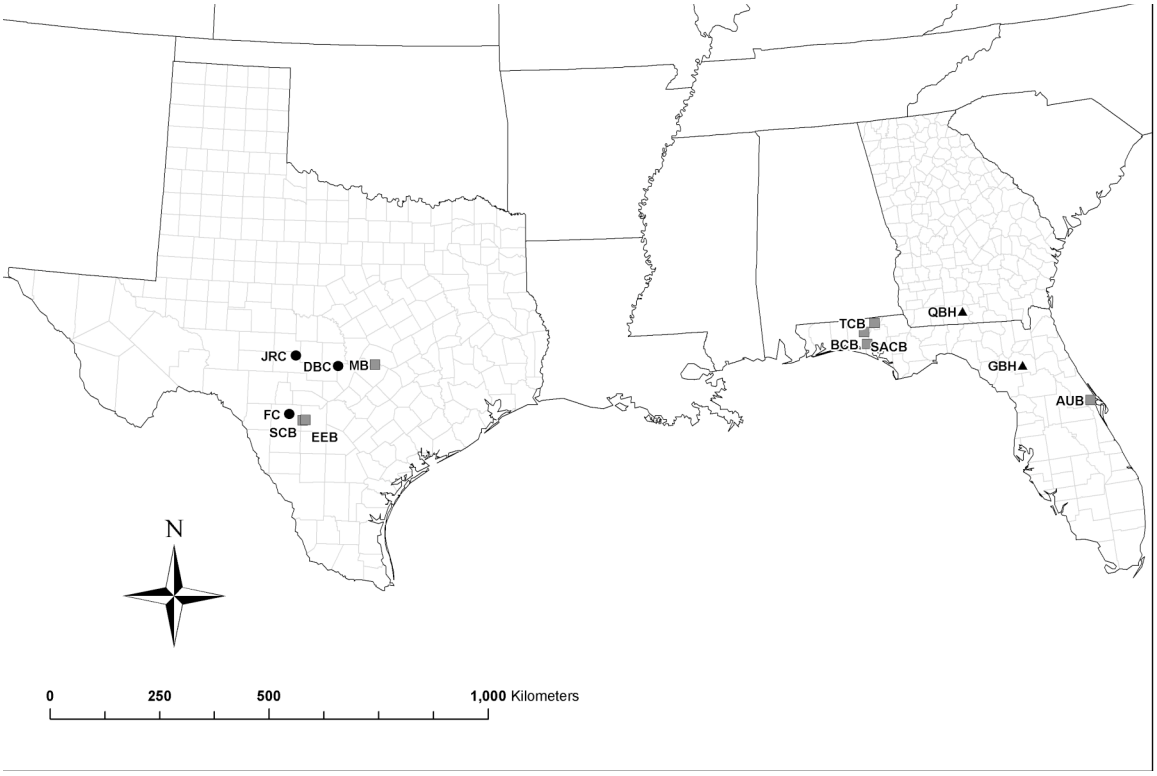

Figure 3.1 Geographic location of all sites used in the combined analysis, which included three cave colonies (black circles; DBC, FC, JRC), seven bridge colonies (gray squares; EEB, SCB, MB, SACB, TCB, AUB, BCB), and two bat house colonies (black triangles; GBH, QBH) of Brazilian free-tailed bats.

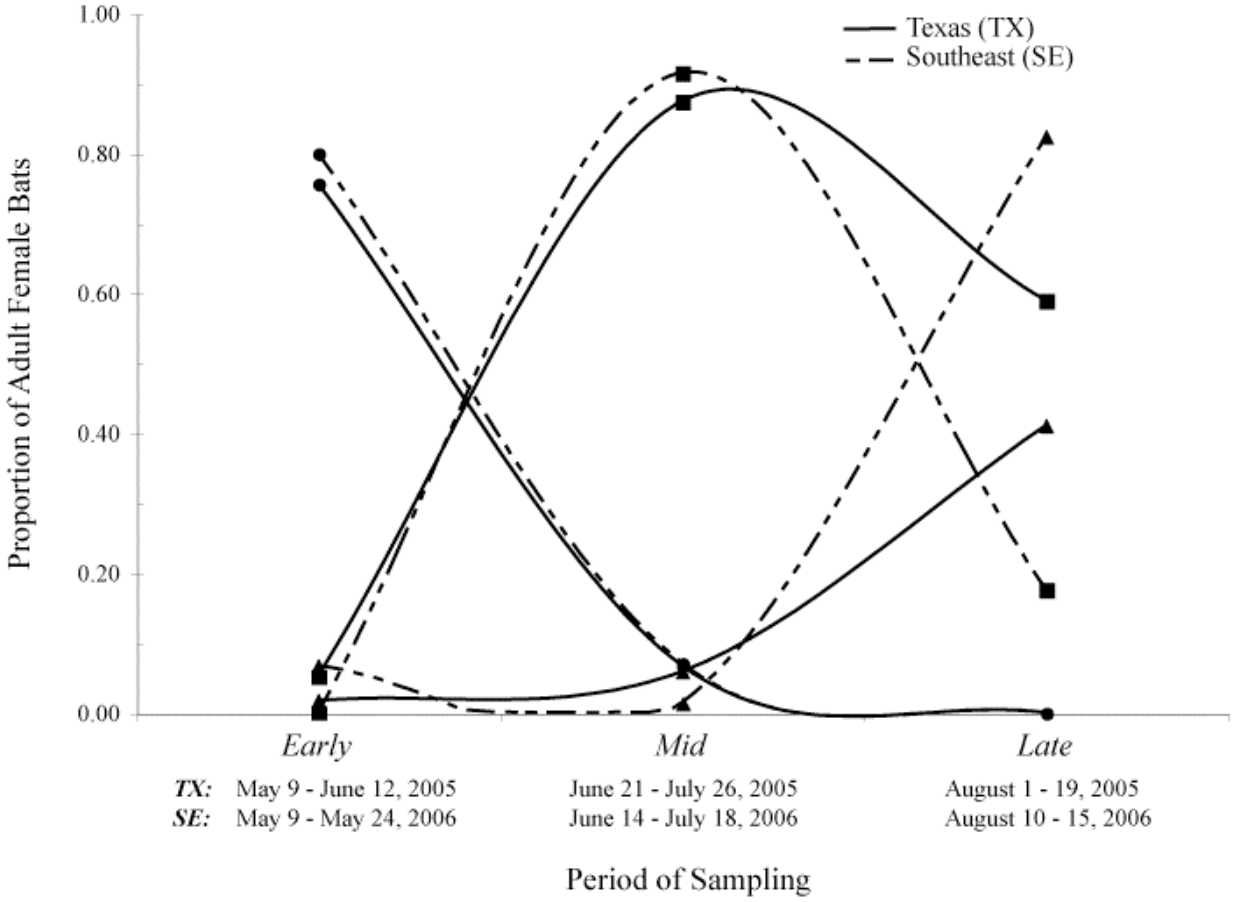

Figure 3.2 The reproductive activity of adult female bats measured across time periods. Exact dates of sampling for different regions and periods are presented. Solid lines represent summary data from six colonies in Texas (DBC, FC, JRC, EEB, SCB, MB), and dashed lines represent six colonies in the southeastern US (AUB, BCB, SACB, TCB, QBH, GBH). Reproductive status was: pregnant (circle), lactating (square), or non-reproductive (triangle). Females of undetermined reproductive status are not shown (n=26).

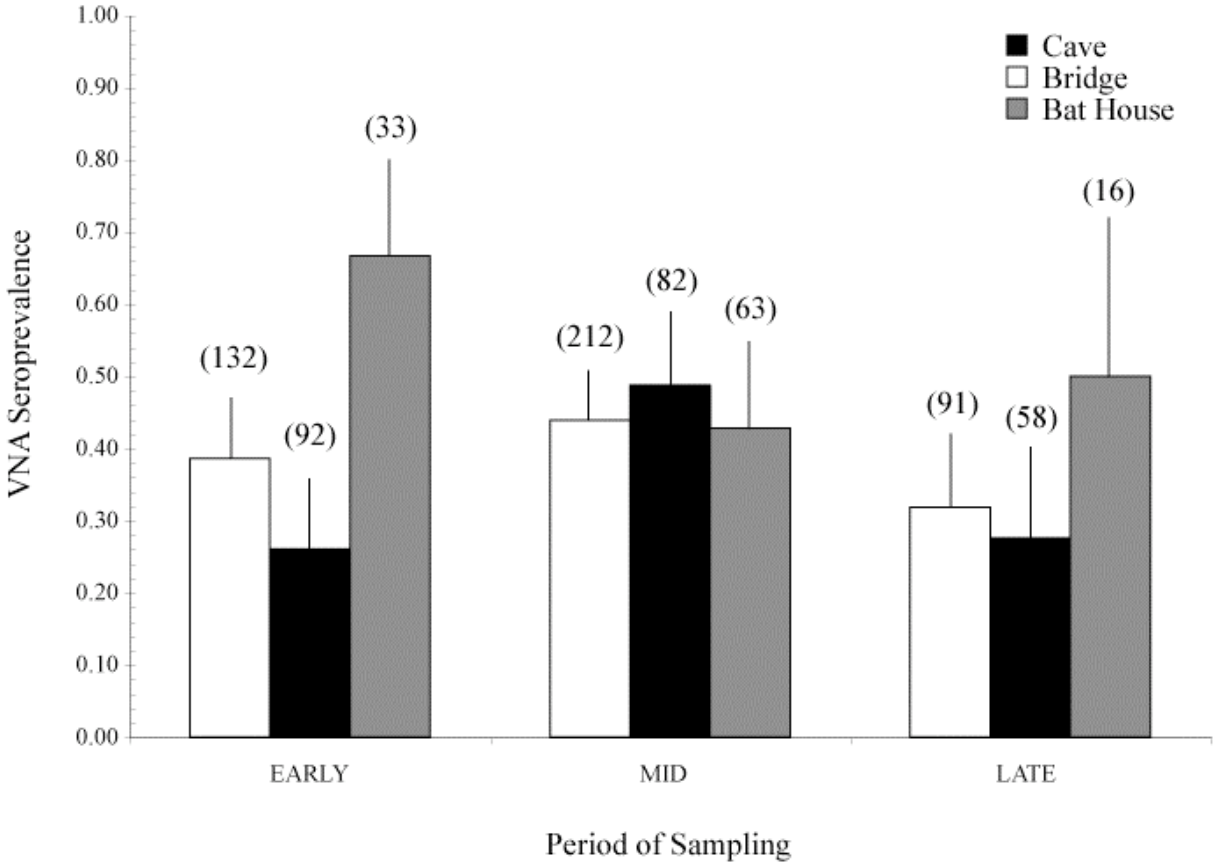

Figure 3.3 Rabies VNA seroprevalence among adult bats from three cave (black; TX - DBC, JRC, FC), seven bridge (white; n=3 – TX – EEB, SCB, MB, n=4 – SE – AUB, BCB, SACB, TCB), and two bat house (gray; SE – QBH, GBH) colonies (n=779). Upper 95% confidence intervals on proportions are shown above histogram bars, and sample sizes are included parenthetically above confidence intervals.

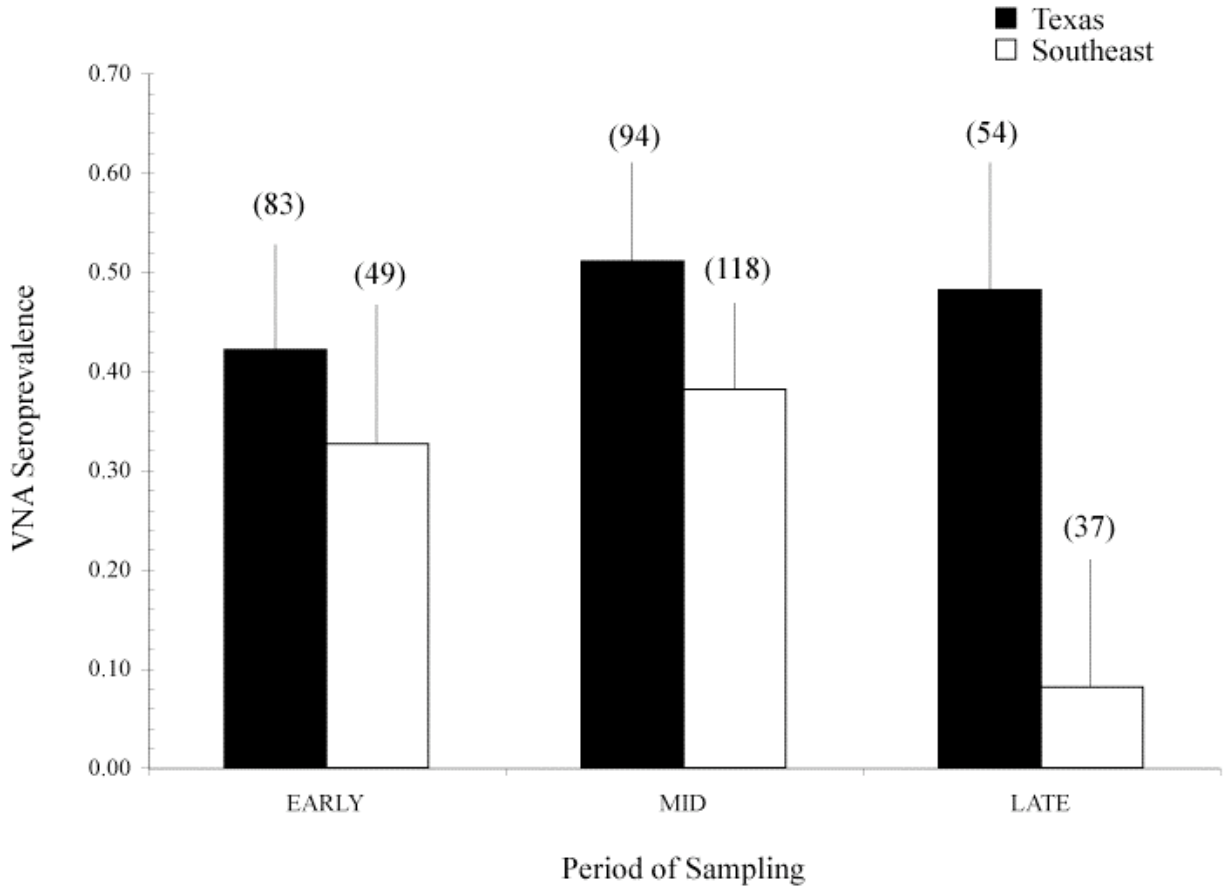

Figure 3.4 Rabies VNA seroprevalence among adult bats from three bridge colonies in Texas (black; EEB, SCB, MB), and four bridge colonies in the southeastern US (white; AUB, BCB, SACB, TCB) (n=284), across time periods. Upper 95% confidence intervals on proportions are shown above histogram bars, and sample sizes are included parenthetically above confidence intervals.

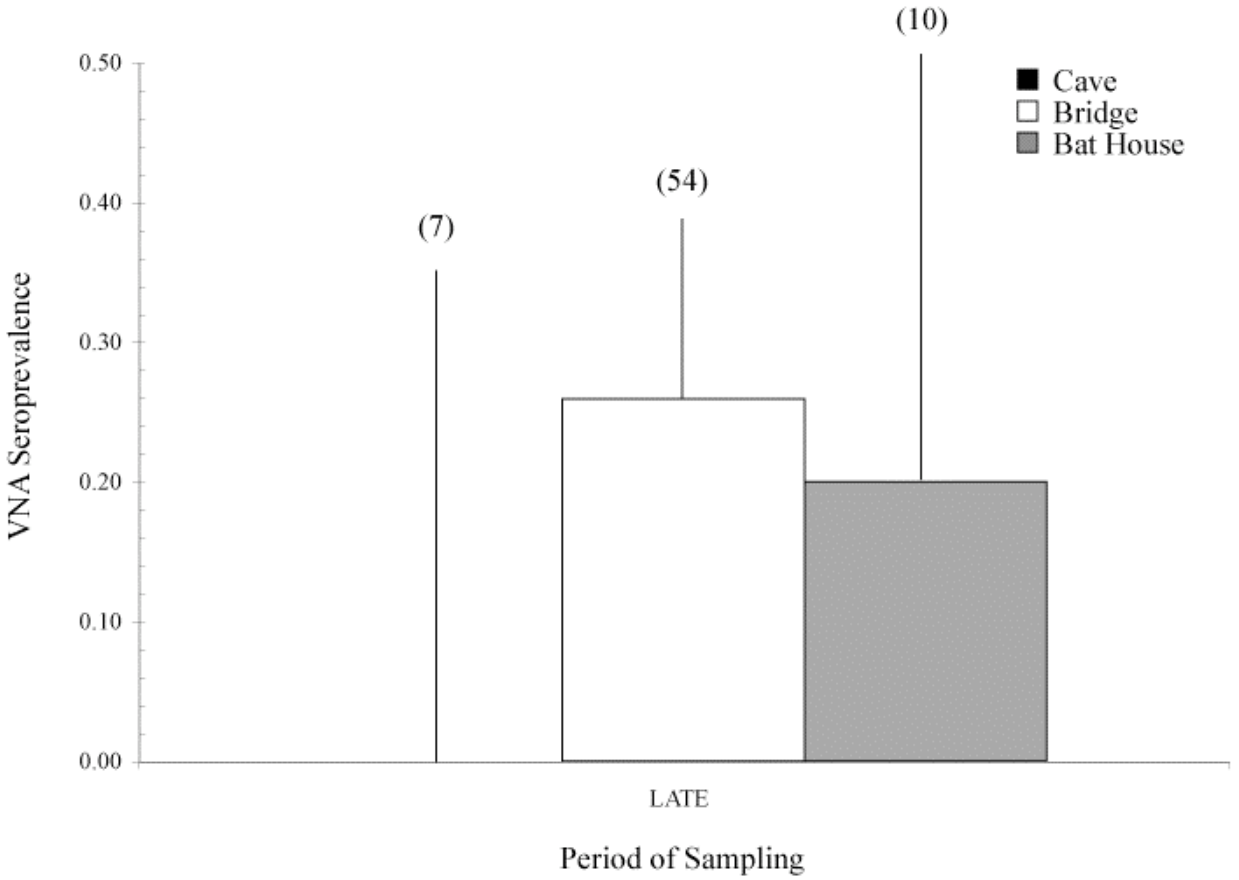

Figure 3.5 Rabies VNA seroprevalence among juvenile bats from three cave (black; TX – DBC, JRC, FC), six bridge (white; n=2 – TX – EEB, MB, n=4 – SE – AUB, BCB, SACB, TCB), and two bat house (gray; SE – QBH, GBH) colonies, during the Late period (n=71). Upper 95% confidence intervals on proportions are shown above histogram bars, and sample sizes are included parenthetically above confidence intervals.

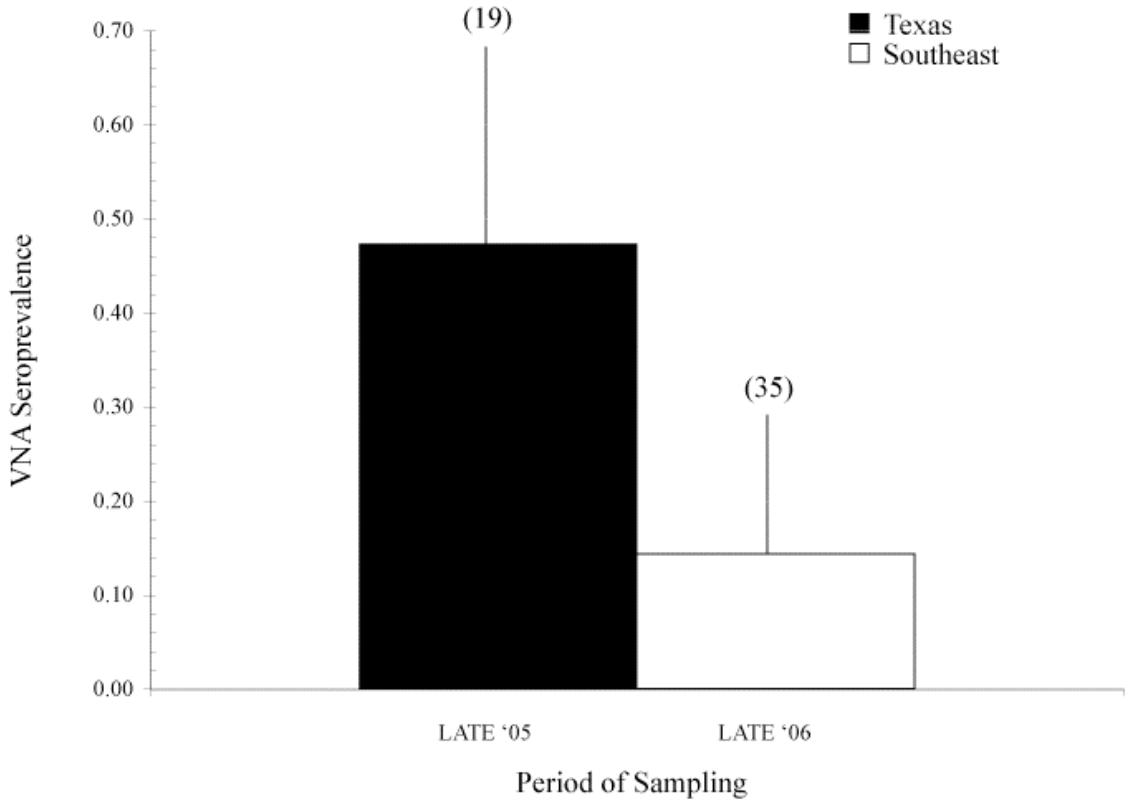

Figure 3.6 Rabies VNA seroprevalence among juvenile bats from two bridge colonies in Texas (black; EEB, MB), and four bridge colonies in the southeastern US (white; AUB, BCB, SACB, TCB), during the Late period (n=54). Upper 95% confidence intervals on proportions are shown above histogram bars, and sample sizes are included parenthetically above confidence intervals.

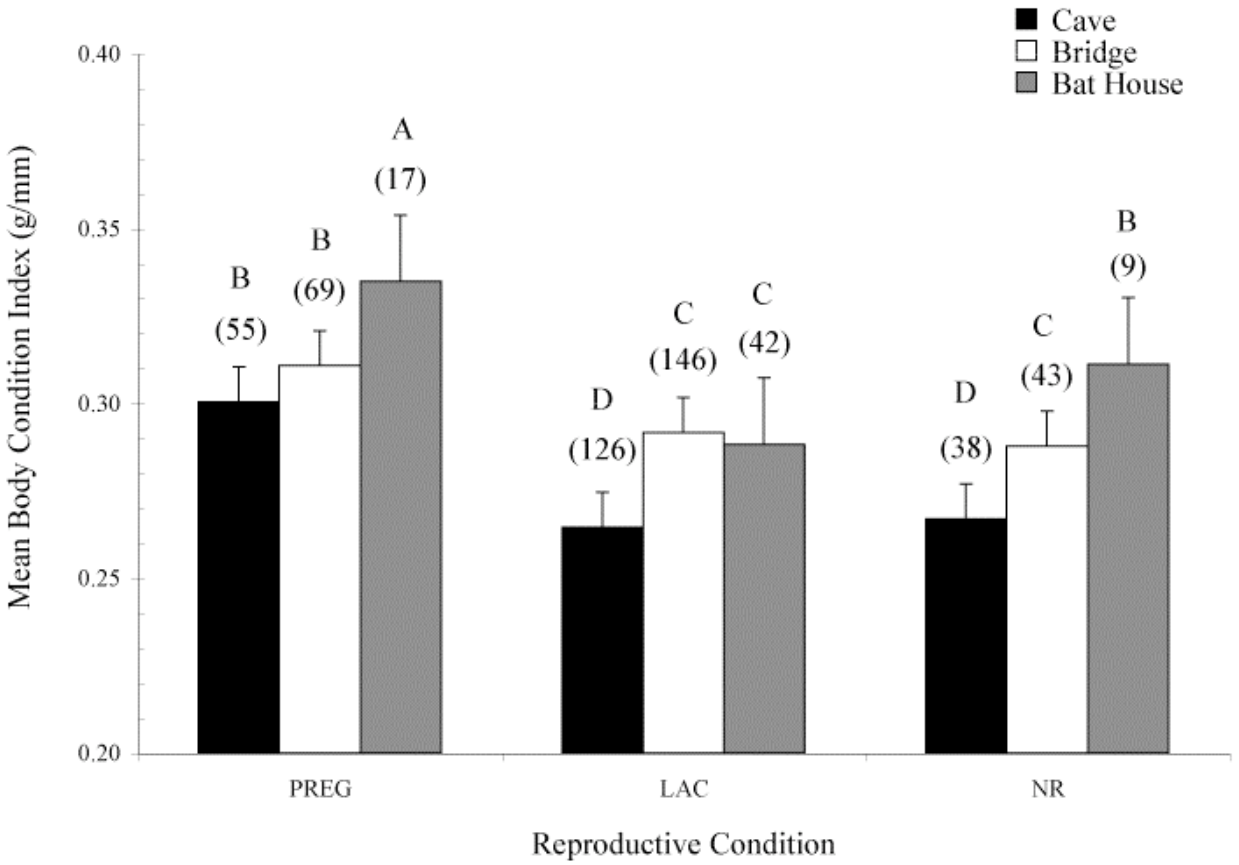

Figure 3.7 Mean (± S.E.) body condition indices among adult female bats, summarized across three cave (black; TX – DBC, JRC, FC), seven bridge (white; n=3 – TX – EEB, SCB, MB, n=4 – SE – AUB, BCB, SACB, TCB) and two bat house (gray; SE – QBH, GBH) colonies, by reproductive status (n=545). Sample sizes are listed parenthetically above each level, and letters above bars denote significant Tukey post-hoc contrasts between levels.

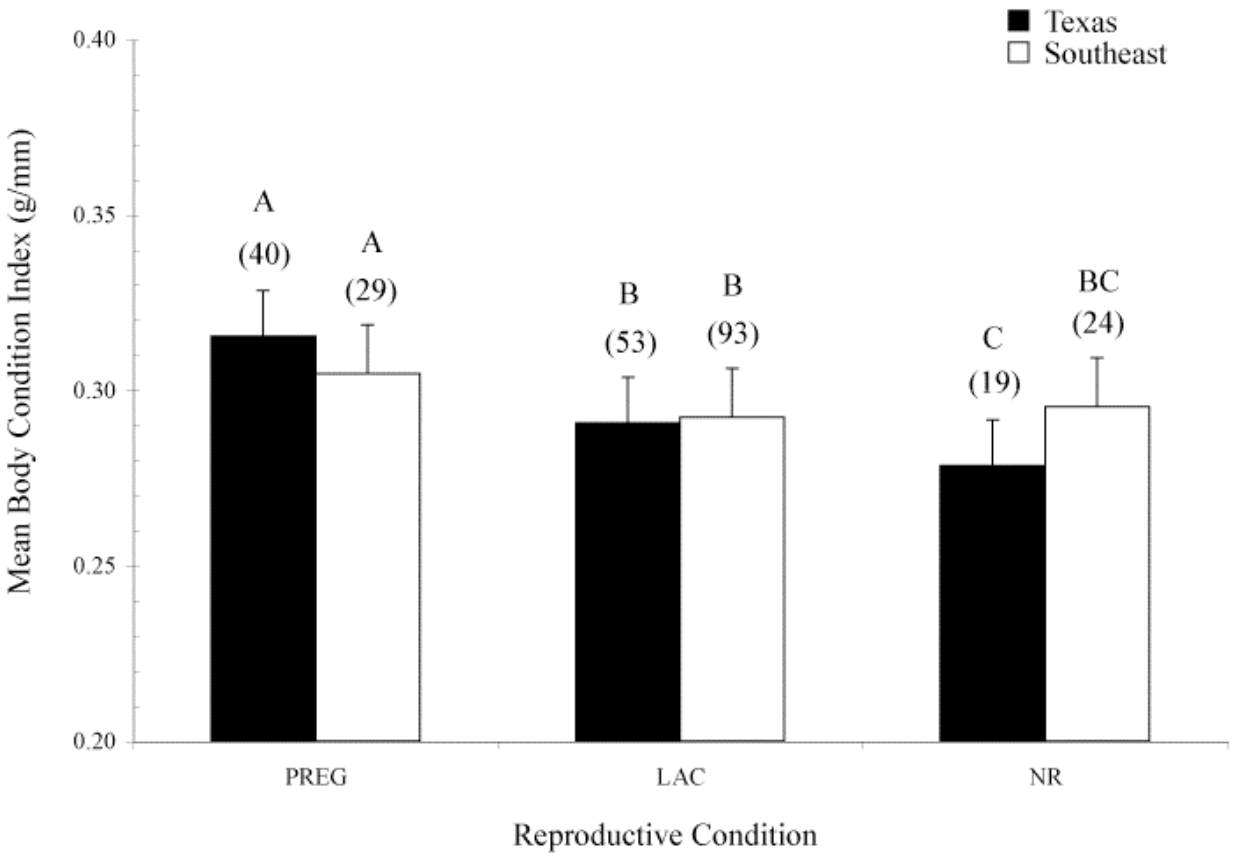

Figure 3.8 Mean (± S.E.) body condition indices among adult female bats, summarized across three bridge colonies in Texas (black; EEB, SCB, MB) and four bridge colonies in the southeastern US (white; AUB, BCB, SACB, TCB), by reproductive status (n=258). Sample sizes are listed parenthetically above each level, and letters above bars denote significant Tukey post-hoc contrasts between levels.

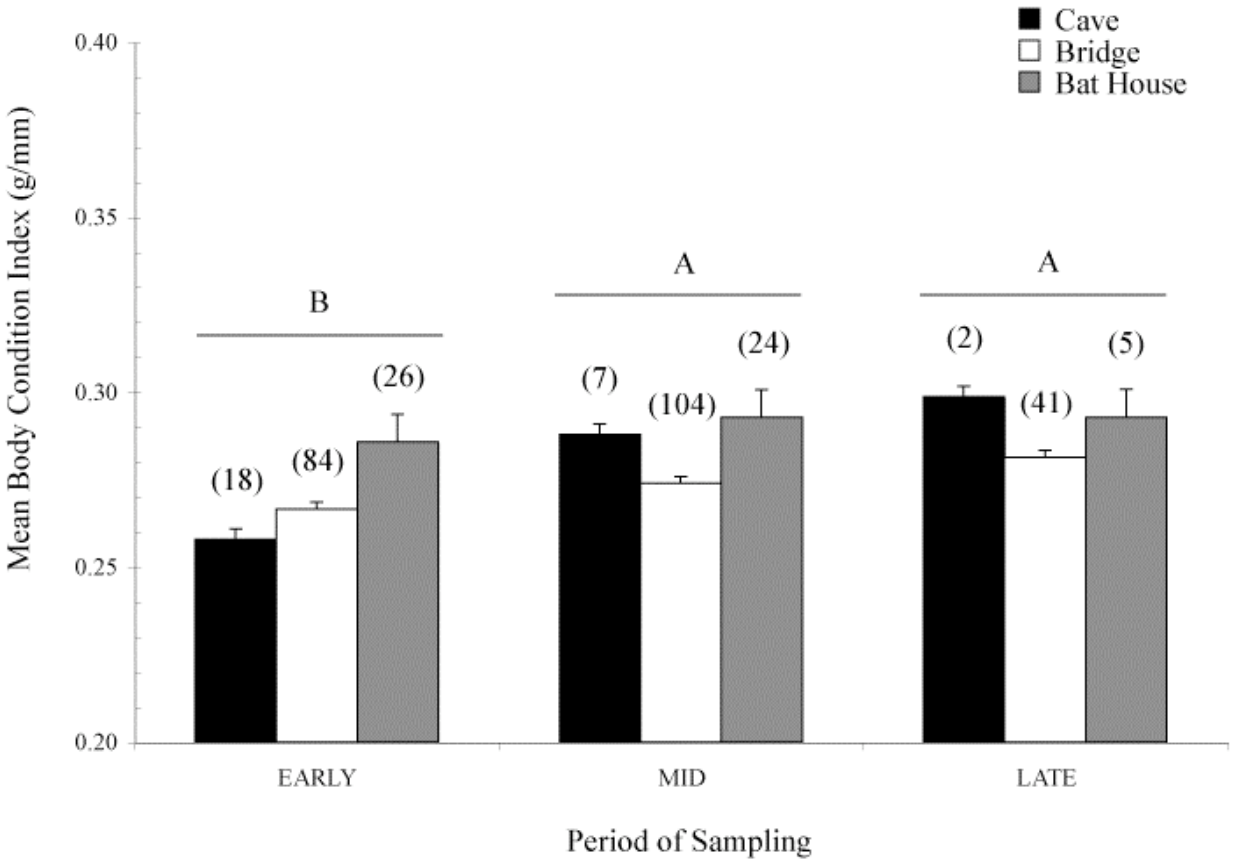

Figure 3.9 Mean (± S.E.) body condition indices among adult male bats from one cave colony (black; TX – FC), six bridge colonies (white; n=3 – TX – EEB, SCB, MB, n=3 – SE – BCB, SACB, TCB) and two bat house colonies (gray; SE – QBH, GBH), across time periods (n=301). Sample sizes are listed parenthetically above each level, and letters above bars denote significant Tukey post-hoc contrasts between levels.

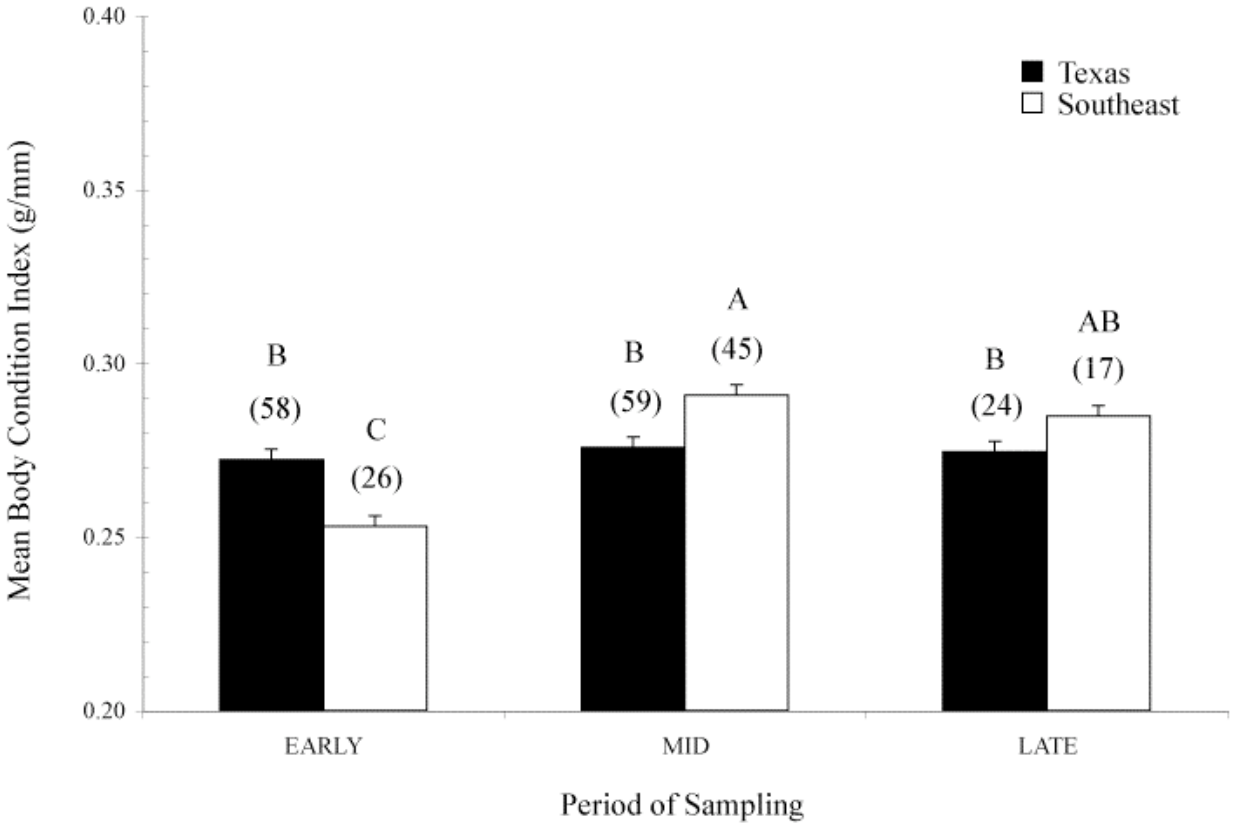

Figure 3.10 Mean (± S.E.) body condition indices among adult male bats from three bridge colonies in Texas (black; EEB, SCB, MB) and three bridge colonies in the southeastern US (white; BCB, SACB, TCB), across time periods (n=229). Sample sizes are listed parenthetically above each level, and letters above bars denote significant Tukey post-hoc contrasts between levels.

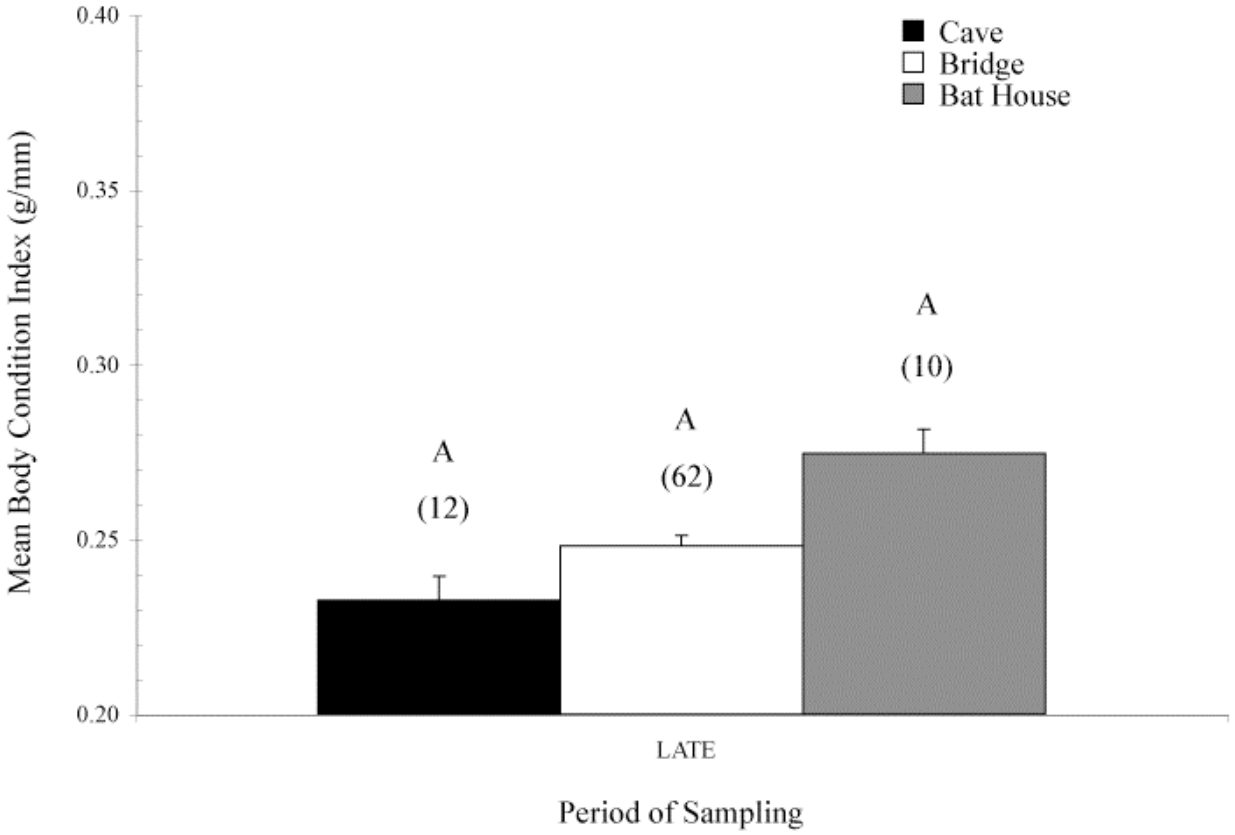

Figure 3.11 Mean (± S.E.) body condition indices among juvenile bats from three cave colonies (black; TX – JRC, DBC, FC), seven bridge colonies (white; n=3 – TX – EEB, SCB, MB, n=4 – SE – AUB, BCB, SACB, TCB) and two bat house colonies (gray; SE – QBH, GBH), during the Late period (n=84). Sample sizes are listed parenthetically above each level, and letters above bars denote significant Tukey post-hoc contrasts between levels.

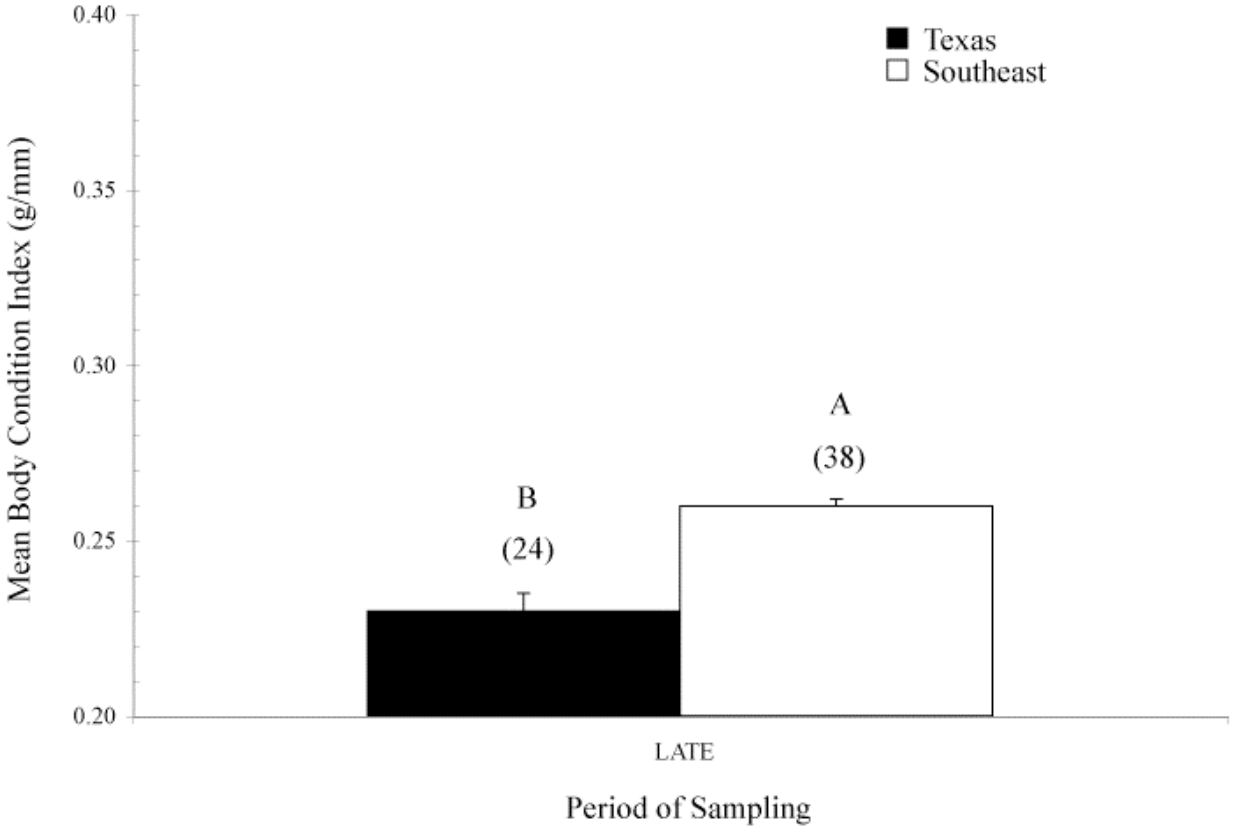

Figure 3.12 Mean (± S.E.) body condition indices among juvenile bats from three bridge colonies in Texas (black; EEB, SCB, MB) and four bridge colonies in the southeastern US (white; AUB, BCB, SACB, TCB), during the Late period (n=62). Sample sizes are listed parenthetically above each level, and letters above bars denote significant Tukey post-hoc contrasts between levels.

Appendix 4

Table 4.1 The experimental results for the COef50 rabies virus infection from three experiments in 42 bats. Dose is median mouse intracerebral doses (MICLD50s).

BAT ID	DOSE (log10)	Inbcubation (days)	dFA[b]	Experiment No.[a]
1864	3.2	13	+	1
1880	3.2	13	+	1
662	3.2	13	+	1
1863	3.2	13	+	1
1858	3.2	14	+	1
481	3.2	15	+	1
478	3.2	15	+	1
480	3.2	15	+	1
1874	3.2	17	+	1
199	3.2	17	+	1
1891	3.2	17	+	1
1894	3.2	17	+	1
1892	3.2	52	+	1
1885	3.2	52	+	1
1879	3.2	52	+	1
1886	3.2	52	+	1
1882	3.2		S	1
1434	3.2		S	1
1889	3.2		S	1
1888	3.2		S	1
476	1.1	19	+	2
435	1.1		S	2
451	1.1		S	2
477	1.1		S	2
445	1.8	17	+	2
444	1.8	29	+	2
443	1.8	31	+	2
446	1.8	37	+	2
436	2.5	12	+	2
704	2.5	12	+	2
452	2.5	13	+	2
453	2.5	13	+	2
1435	1.8	17	+	3
1849	1.8	17	+	3
1433	1.8		S	3
447	2.5	21	+	3
448	2.5		S	3
1879	2.5		S	3
450	3.2	12	+	3
1887	3.2	15	+	3
1861	3.2	15	+	3
1850	3.2	17	+	3

a Experiment 1 data are taken from Jackson et al. 2008.
b dFA results are positive (+) or survived (S; no dFA).

Table 4.2 The experimental results for the PAef137 rabies virus infection from four experiments in 61 bats. Dose is median mouse intracerebral doses ($MICLD_{50}$s).

BAT ID	DOSE (log10)	Inbcubation (days)	dFA[a]	Experiment No.
487	-0.1	135	+	4
486	-0.1		S	4
488	-0.1		S	4
491	-0.1		S	4
492	-0.1		S	4
483	0.9		C *(day 12)*	4
464	0.9		S	4
484	0.9		S	4
485	0.9		S	4
1892	0.9		S	4
462	1.9	32	+	4
1862	1.9	38	+	4
460	1.9		S	4
461	1.9		S	4
494	1.9		S	4
1883	2.9	15	+	4
454	2.9	26	+	4
455	2.9	26	+	4
456	2.9		S	4
457	2.9		S	4
497	3.9	27	+	4
440	3.9		S	4
442	3.9		S	4
498	3.9		S	4
1881	3.9		S	4
84	1.9	13	+	5
82	1.9	19	+	5
95	1.9	140	+	5
83	1.9		S	5
79	1.9		S	5
87	2.9	15	+	5
96	2.9	22	+	5
88	2.9		S	5
89	2.9		S	5
90	2.9		S	5
74	3.9	17	+	5
76	3.9	18	+	5
77	3.9	19	+	5
75	3.9		C *(day 113)*	5
78	3.9		S	5
71	4.9	13	+	5
490	4.9	16	+	5
467	4.9		S	5
63	4.9		S	5
68	4.9		S	5
32	2.9	20	+	6
58	2.9	26	+	6
34	2.9	26	+	6
37	2.9		C *(day 43)*	6
49	2.9		C *(day 122)*	6
35	2.9		S	6
36	2.9		S	6
65	2.9	5	+	7
16	2.9	38	+	7
11	2.9	50	+	7
30	2.9	61	+	7
21	2.9		C *(day 76)*	7
17	2.9	91	+	7
27	2.9	115	+	7
465	2.9		S	7
18	2.9		S	7

a dFA result is positive (+), censored (C; dFA neg), or survived (S; no dFA).

Figure 4.1 The geographic range of *Eptesicus fuscus* in North America (Hall, 1981), with the overlay of morphological subspecies classifications (Burnett, 1983a, b).

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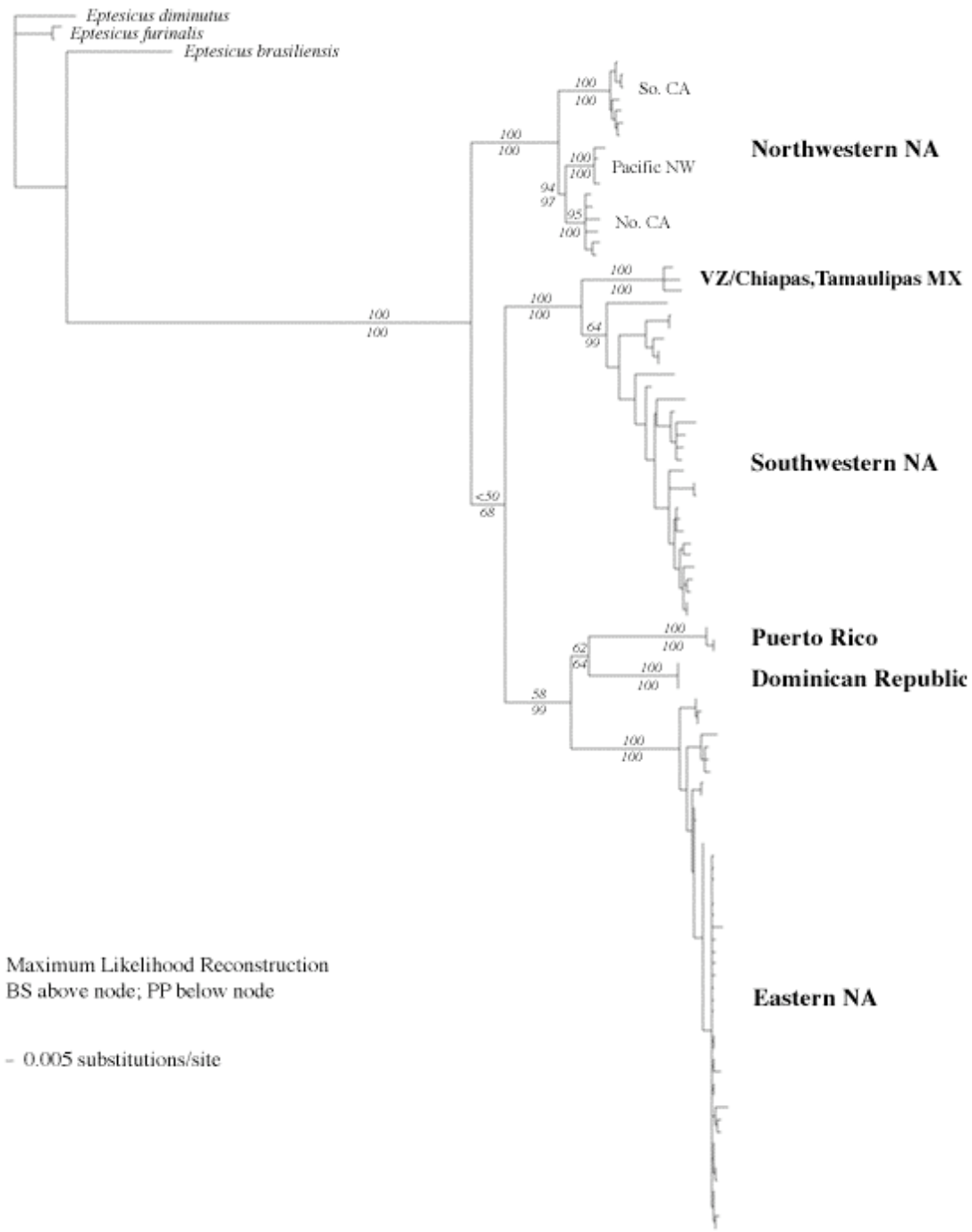

Figure 4.2 The maximum likelihood phylogeny of *Eptesicus fuscus* across North America (n=102), from concatenated mitochondrial ND2 and control region sequence data (0.9Kb) (Turmelle et al., *In Prep.*).

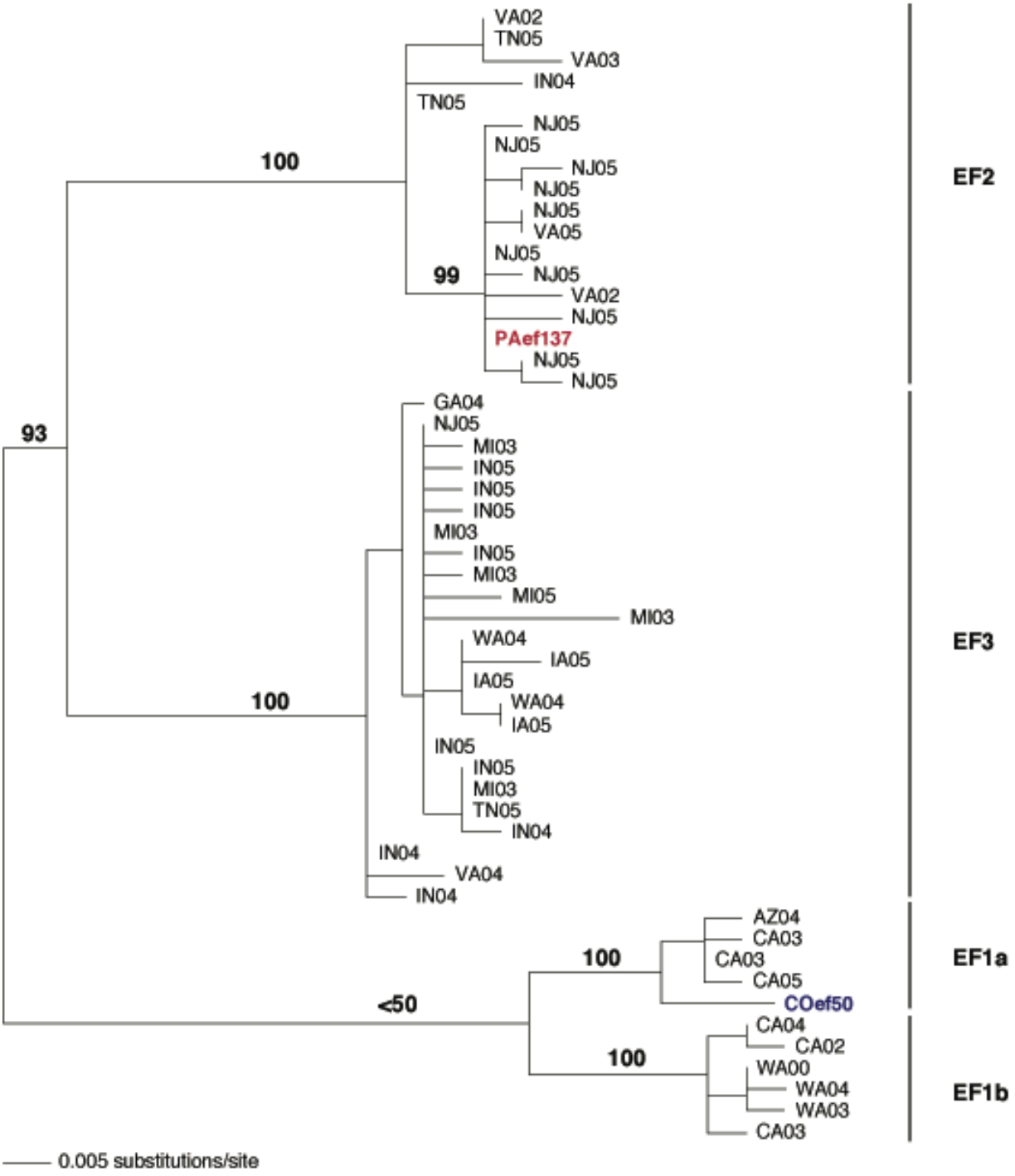

Figure 4.3 Phylogeny of rabies virus nucleoprotein sequence variants from *Eptesicus fuscus* across the United States (sequences provided by D. Streicker). State abbreviations are given for each sample, and the last two digits for the year of specimen submission follow the state abbreviation. Posterior probabilities are shown above selected nodes.

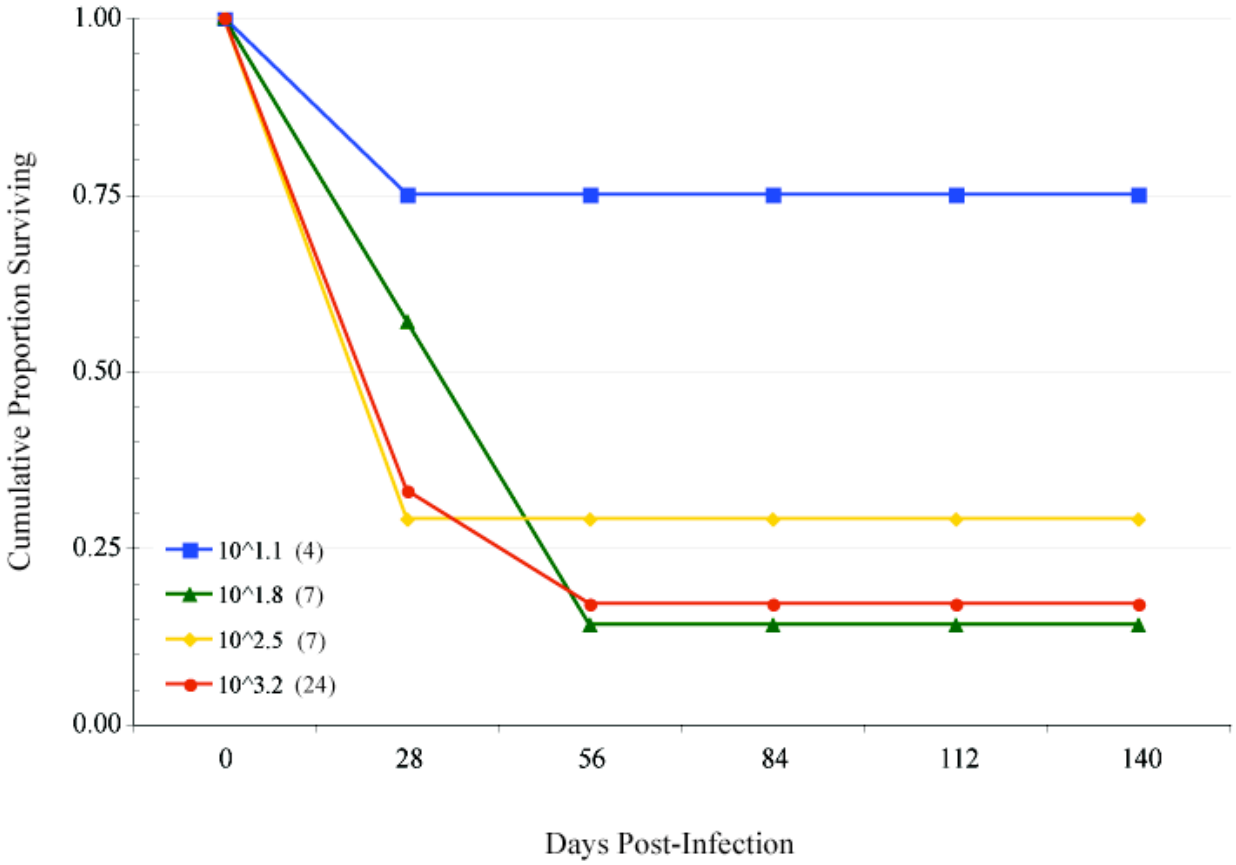

Figure 4.4 The survival table for the COef50 RABV isolate through 140 days post-infection. Sample sizes for each dose are listed parenthetically in the legend inset.

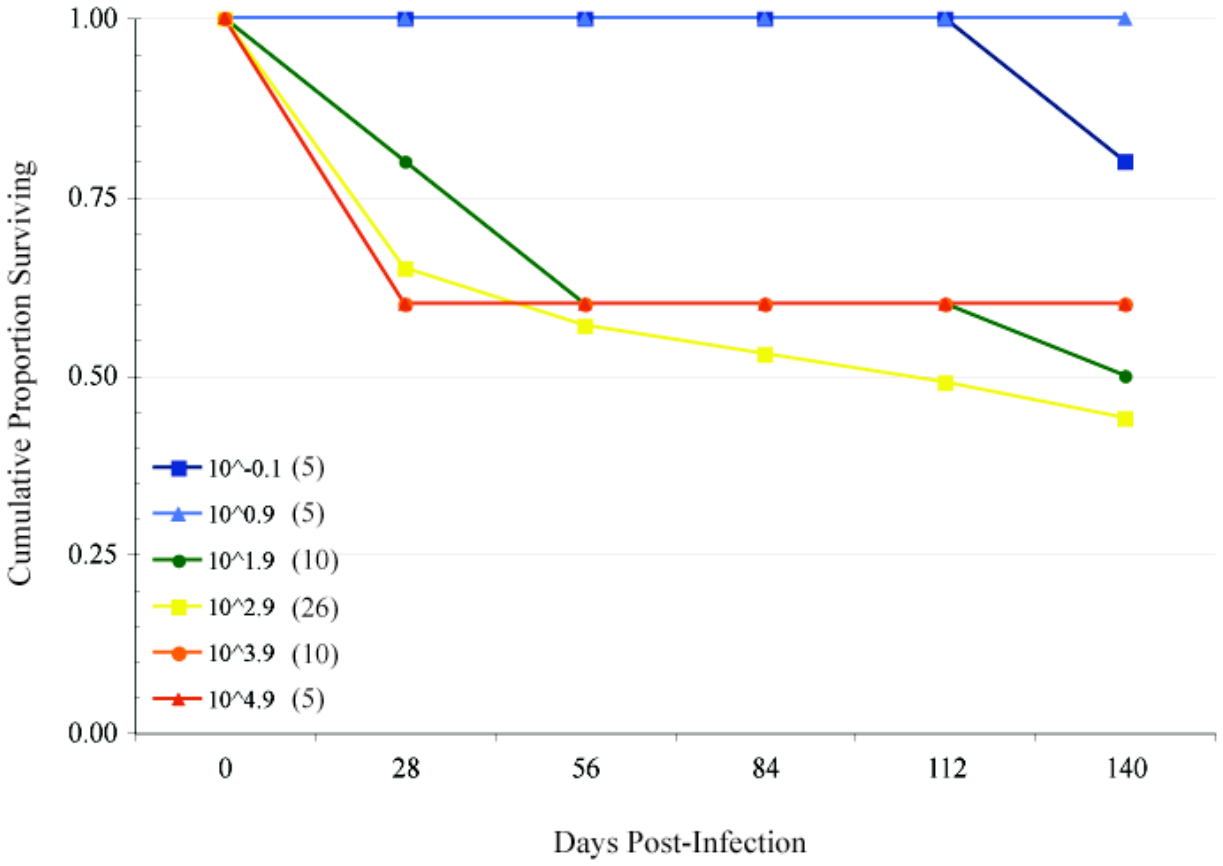

Figure 4.5 The survival table for the PAef137 RABV isolate through 140 days post-infection. Sample sizes for each dose are listed parenthetically in the legend inset.

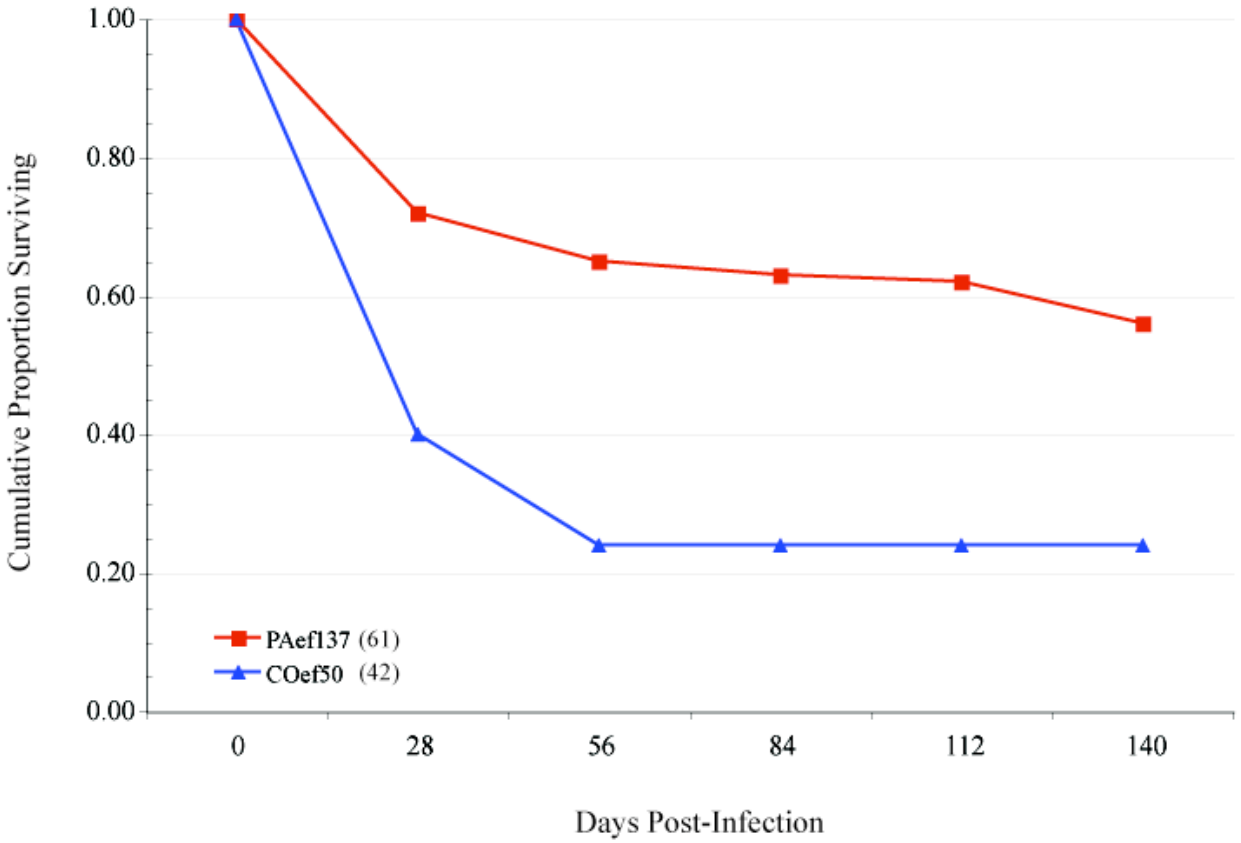

Figure 4.6 The survival table comparing the PAef137 and COef50 isolates, summarized across all doses, through 140 days post-infection. Samples sizes for each isolate are listed parenthetically in the legend inset.

VITA

Amy Susanne Turmelle was born in Manchester, NH on October 4, 1980. She graduated from Londonderry High School and went on to pursue her Bachelor's degree in Biology at Boston University (BU). Amy completed her Bachelor of Arts degree in 2002, and then moved to New York City for an internship at the American Museum of Natural History (AMNH). In 2004, she entered the doctoral program at the University of Tennessee to pursue research on rabies in bats. Following completion of her doctorate degree in Ecology and Evolutionary Biology in 2009, Amy will begin a postdoctoral position at the Centers for Disease Control and Prevention to continue studying emerging infectious diseases in bats.