DISSERTATION

BATS AS RESERVOIR HOSTS: EXPLORING NOVEL VIRUSES IN NEW WORLD BATS

Submitted by

Ashley Malmlov

Department of Microbiology, Immunology, and Pathology

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Doctoral Committee:

Advisor: Tony Schountz

Richard Bowen
Page Dinsmore
Kristy Pabilonia

ABSTRACT

BATS AS RESERVOIR HOSTS: EXPLORING NOVEL VIRUSES IN NEW WORLD BATS

Order Chiroptera is oft incriminated for their capacity to serve as reservoirs for many high profile human pathogens, including Ebola virus, Marburg virus, severe acute respiratory syndrome coronavirus, Nipah virus and Hendra virus. Additionally, bats are postulated to be the original hosts for such virus families and subfamilies as *Paramyxoviridae* and *Coronavirinae*. Given the perceived risk bats may impart upon public health, numerous explorations have been done to delineate if in fact bats do host more viruses than other animal species, such as rodents, and to ascertain what is unique about bats to allow them to maintain commensal relationships with zoonotic pathogens and allow for spillover. Of particular interest is data that demonstrate type I interferons (IFN), a first line defense to invading viruses, may be constitutively expressed in bats. The constant expression of type I IFNs would hamper viral infection as soon as viral invasion occurred, thereby limiting viral spread and disease. Another immunophysiological trait that may facilitate the ability to harbor viruses is a lack of somatic hypermutation and affinity maturation, which would decrease antibody affinity and neutralizing antibody titers, possibly facilitating viral persistence.

In 2009 and 2010 two novel influenza A viruses (IAV) were discovered via qRT-PCR using pan-influenza primers in New World bat species. Given the unique hemagglutinin (HA) and neuraminidase (NA) glycoproteins compared to those already recognized, the two viruses were classified as H17N10 and H18N11. H17N10 IAV genome was discovered in rectal swabs collected from little yellow-shouldered bats in Guatemala, and H18N11 IAV genome was discovered in a rectal swab and gastrointestinal tract of a flat-faced fruit bat from Peru. The entire sequences for both viruses were identified using next generation and Sanger sequencing, but the virus was never isolated from wild bat populations. Both viruses differ from canonical IAVs in that the HA does not bind to the host sialic acid receptor and the

function of NA remains undetermined. Given the divergence from other IAVs, the attention bats receive as reservoir hosts, and the lack of isolation of wild virus, H17N10 and H18N11 remain shrouded in mystery. Reverse genetics was used to rescue both viruses and we performed experimental infections in Jamaican fruit bats (*Artibeus jamaicensis*), drawing upon the colony housed at Colorado State University. Evidence for H17N10 infection could not be elicited after inoculation, and thus it was concluded that Jamaican fruit bats may not be susceptible. Bats inoculated with H18N11 seroconverted (determined by ELISA), had viral RNA detected in rectal but not oral swabs by qRT-PCR, and had viral RNA present in the length of the gastrointestinal tract detected by qRT-PCR. Hematoxylin and eosin stain used to characterize histopathology revealed minimal pathology that was predominately localized to the gastrointestinal tract in the form of neutrophilic, plasmacytic and lymphocytic cellular infiltration. Furthermore, two naïve transmission bats were exposed to inoculated bats and demonstrated seroconversion and viral RNA detected in rectal swabs by qRT-PCR. Results demonstrate that Jamaican fruit bats are susceptible to H18N11 and indicate transmission occurs fecal-orally. Tissue tropism is for the gastrointestinal tract. These data recapitulate transmission and tissue tropism as seen in the reservoir of IAV, water fowl, and low-pathogenic avian influence viruses in gallinaceous birds. However, this does not indicate that bats may be a reservoir for influenza viruses as H18N11 is not known to cause disease in humans and is highly divergent from other IAVs. More likely, this demonstrates an early divergence of H18N11 from other IAVs and a long-lived co-evolution between the host and the virus. Further investigation of H18N11 may provide information on relationships between bats and their viromes, which is of great importance given so many bat species harbor highly pathogenic zoonotic viruses.

The impact of Zika virus (ZIKV) on the New World has been great—infecting more than 200,000 people and manifesting in some patients as severe neurological complications including microcephaly in infected fetuses and Guillain-Barré syndrome. An enzootic cycle is implicated as an important part of viral ecology, yet little is known about this cycle. Historically, different bat species demonstrated experimental susceptibility to ZIKV as they seroconvert, have neurological disease, viremia and ZIKV

positive tissues. Jamaican fruit bats are endemic to a region that temporally overlaps with the distribution of ZIKV in the Americas and Caribbean. We sought to identify if these bats were susceptible to ZIKV and conducted a time course study to delineate progression of viral infection and pathophysiology. ELISAs were used to identify seroconversion. Quantitative RT-PCR and immunohistochemistry were used to determine tissue tropism, and hematoxylin and eosin stain was used to characterize histopathology. Jamaican fruit bats seroconverted post-inoculation with ZIKV. Evidence for virus was found by qRT-PCR in the brain and urine for ZIKV infection in some bats. IHC revealed positive testes, brain, lung, and salivary gland and a trend toward mononuclear cells, macrophage and fibroblast viral tropism. Histopathology was seen in the brain, testes and salivary gland. Based on these data it was concluded that Jamaican fruit bats could become infected with ZIKV. These bats may have a role in viral ecology.

Tacaribe virus (TCRV) was isolated in the 1950s from wild artibeus bats captured in Trinidad, West Indies. The initial characterization of TCRV suggested that artibeus bats were natural reservoir hosts. However, nearly 60 years later experimental infections of Jamaican fruit bats resulted in fatal disease or clearance, suggesting this species is not a reservoir host. To further evaluate the TCRV reservoir host status of artibeus bats, we captured bats of six species in Trinidad for evidence of infection. Bats of all four frugivorous species had antibodies to TCRV nucleocapsid, whereas none of the insectivore or nectarivore species did. Many flat-faced fruit bats (*A. planirostris*) and great fruit bats (*A. literatus*) were seropositive by ELISA and western blot to TCRV nucleocapsid antigen, as were two of four Seba's fruit bats (*Carollia perspicillata*) and two of three yellow-shouldered fruit bats (*Sturnira lilium*). Serum neutralization tests failed to detect neutralizing antibodies to TCRV from these bats. TCRV RNA was not detected in lung tissues or lung homogenates inoculated onto Vero cells. These data suggest that TCRV or a similar arenavirus continues to circulate among fruit bats of Trinidad but there is no evidence of persistent infection, suggesting artibeus bats are not reservoir hosts.

In order to make species specific reagents and expand laboratory research on Jamaican fruit bats, *de novo* assembly of the Jamaican fruit bat genome was performed with Soapdenovo2. The final genome size was 1.4 kilobases with 20x coverage.

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1.1.1: Bat Evolution

Approximately 65.5 million years ago (Ma) there was an abrupt decline in species diversity that marked the Cretaceous-Paleogene (K-Pg) boundary. It is generally accepted that the mass die-off was from the direct impact and subsequent environmental changes of an asteroid, ~10 km in diameter, that collided with Earth in what is now the Gulf of Mexico (1). Mammals as a class existed prior to this juncture, alongside dinosaurs. Molecular clocks estimate that placental mammals split from the basal group of eutherians 80 to 100 Ma during the mid to late Cretaceous period, prior to the K-Pg boundary (2). Based on combined phenomic and molecular parsimony, it is hypothesized that a single placentalial lineage survived the K-Pg event and this lineage radiated into the 18 extant eutherian orders during the early Paleocene, 66 to 56 Ma, including order Chiroptera (3).

Geographical ancestral reconstructions suggest that bats originated in Laurasia, possibly North America (4). Phylogenetic analysis of cytochrome c of 648 bat species places the origin of the order at 58.9 Ma, during the Cenozoic Era (5). Another study estimates a common ancestor of 64 Ma (4). Yet another study used molecular analysis to support the hypothesis that bats evolved from a horse lineage approximately 88 Ma (6). The oldest, most complete, full flight bat fossils date back 52.5 Ma (7), found in what is now Wyoming. However, it is generally accepted that the fossil record is askew from the actual age of species due to a limited number of fossils found. The bat fossil record is sparse with an estimated 61% to 88% of the fossil record unaccounted for (4, 8). Regardless of any incongruence in dating the age of bats, the scientific consensus is that they are an ancient order of mammals.

There are currently more than 1,100 bat species on every continent except Antarctica. Bat species comprise 20% of mammalian diversity (9). Within the order Chiroptera there are two suborders;

Yinpterochiroptera and Yangochiroptera (10). Yinpterochiroptera are then divided into six families comprised of Old World bats (4, 11). Yangochiroptera are divided into 12 families consisting of both New World and Old World bat species (4, 11). By the end of the Eocene (33.9 Ma), all 18 extant bat families had been established, highlighting the age of the order and suggesting that each bat family is distinct from one another as separated by time (4). Family *Phyllostoma* is comprised of New World leaf-nosed bats and is placed within Yinpterochiroptera. It is estimated to have split from its sister lineage 28.5 Ma, with the oldest fossil dated back to 16 Ma (5).

1.1.2: Family *Phyllostoma*

Within the family of *Phyllostoma* is the genus *Artibeus*. There are 13 species of *Artibeus* bats with molecular clock analysis dating the crown ancestor at ~5.1 Ma. The Jamaican fruit bat (*Artibeus jamaicensis*), which is the focus of much of this work, is estimated to have split from a common ancestor 2.5 Ma (12). At Loltun Cave in the Yucatan Peninsula, 1,522 subfossil bones were found in late Pleistocene and Holocene sediment, placing the oldest fossils at 2.5 Ma to 11.5 thousand years ago, corroborating molecular dating (13).

Morphological phylogeny places the distribution of Jamaican fruit bats from Mexico south to Ecuador, following the boundary of the Andes Mountains. On the east side of the Andes Mountains they populate Bolivia, Paraguay and Brazil north into the Caribbean Islands and the Florida Keys. Many species of the *Artibeus* genus look similar, and the species inclusion parameters allows for broad morphology (13). When phylogenetics are combined with morphology, there is evidence that the Andes Mountains serve as a natural barrier for Jamaican fruit bats, limiting the South American distribution to the west side of the mountains. On the east side of the Andes Mountains a morphologically similar species is found; the flat-faced fruit-eating bat (*A. planirostris*) (12).

The flat-faced fruit-eating bat also inhabits the southern Caribbean Islands of Trinidad/Tobago, Grenada, and the Grenadines. Where the Caribbean Islands are concerned phylogenetic criterion identify Jamaican fruit bats on the northern islands as far south as the Grenadines and Grenada. However, the Jamaican fruit bats are not as abundant as flat-faced fruit-eating bats on the islands they co-habitat (Grenadines and Grenada). Additionally, there is a third species present on these two islands, and islands immediately north. This third species, *A. schwartzi*, may be a hybrid between the Jamaican fruit bat and the flat-faced fruit-eating bat based on mitochondrial DNA sequences. If *A. schwartzi* is a hybrid then this is one of the few documented cases of mammalian natural hybridization (12). This information emphasizes how little is known about bat phylogeny and biodiversity, particularly as it pertains to *Artibeus* bats.

Jamaican fruit bats occupy a variety of habitats including humid tropical forests, drier tropical forests, and areas populated and modified by humans—emphasizing the species adaptability. They roost in dense foliage, hollow trees, caves, buildings, and will make temporary leaf tents. They are generalist frugivorous but prefer figs, and will selectively eat leaves high in protein. The juice of the fig serves as a staple in their diet with much of the fruit fiber ignored. It is published that this form of eating satisfies their water intake so they do not have to drink water (13); however, this author has observed bats of the *Artibeus* genera consuming water in the wild (unpublished data).

In the wild the lifespan of Jamaican fruit bats is estimated to be nine years. Females are sexually matured at 8 months and males at 12 months. Females are reported to have a bimodal polyestrous breeding cycle with peak breeding season occurring at the end of the wet season and parturition occurring in the dry season; although different populations breed year-round with lactating or pregnant females being trapped throughout the year. Indeed, the captive colony at Colorado State University seems to breed year-round. Gestation is 3.5 to 4 months but can be delayed for 7 months. Pups nurse for 15 days,

have complete dentition at 40 days and fledging begins at 50 days. By 80 days pups are at their adult weight.

Jamaican fruit bats roost in harems that contain approximately 14 females and two harem males. Harem females stake out the optimal territory for mating while males dominate and defend resources. Female sub adults leave the harem together to roost with a different harem. Males tend to stay with their natal harems. Sexually mature satellite males are pushed to the edges of the roosting space and may group with other satellite males and non-reproductive females (13).

1.2: Bats as Reservoirs and Original Hosts for Viruses

Bats are notorious for being reservoirs of zoonotic infectious disease, particularly high-profile viruses such as lyssa-, filo-, henipa-, and coronaviruses. Additionally, bats may be the original hosts for virus genera *Lyssavirus*, *Hepacivirus*, and *Pegivirus*, and virus family *Paramyxoviridae* (14-25).

1.2.1: *Lyssavirus*

Lyssa is the Greek goddess of rage and furry. The genus *Lyssavirus* includes 14 species that may cause rabies-like disease characterized by encephalomyelitis, including rabies virus (RABV) (26). Lyssaviruses are rod shaped and enveloped, with a single-stranded, non-segmented, negative sense RNA genome (27). RABV is the best-characterized lyssavirus and while difficult to ascertain the global human mortality incidence, it causes tens of thousands of deaths annually. RABV infection is near always fatal (28). The virus can infect all mammal species, but is predominately carried and transmitted by dogs in the Eastern Hemisphere, and bats, dogs, fox, raccoons, skunks, and other wild mesocarnivores in the Western Hemisphere (26). The origin of the strains circulating in wild mesocarnivores (with the exception of skunks) is a dog-derived variant; however, there are more than 30 RABV variants associated with more than 20 bat species in the Americas (15). In the Western Hemisphere, human exposure to

RABV is most often due to bats, and the incidence of human cases of bat associated RABV in North America is 2.2-6.7 cases per billion people per year (15, 27).

Sequence data of modern RABV indicate the age of the virus to be 1000 to 1500 years old (15, 27), but there are historical documents that imply the disease was recognized in ancient Mesopotamia in 1800 BC (15, 29). Old World bat species are most likely the origin for lyssaviruses, and responsible for global dispersion as they are the principle hosts of the 14 extant virus species world-wide (14, 15). However, presently, only New World bats harbor RABV, and serve as the dominant mammalian host in the Western Hemisphere. Non-RABV lyssaviruses exist in bats in the Eastern Hemisphere (27, 30). Historically, RABV switched hosts a minimum of twice, once from Old World bats into New World bats, and once from Old World bats into dogs in Europe or Asia. Phylogenetics dates the introduction of RABV lineages in the Western hemisphere to 1400 years ago, before the European exploration of the Americas. Historical records support phylogenetic dating, connecting human cases of disease in the Americas to bats but not to dogs. The dog variant, which is currently the most prevalent strain in the Old World, was most likely introduced to the New World in the 17th century when advances in technology shortened the time it took to travel to the Americas from months to weeks. RABV incubation period is three to eight weeks, and so travel that consists of months could serve as an inadvertent quarantine period and may have limited the spread of Old World canine variants to the New World. The first recorded dog epizootic occurred in 1709 in Mexico City, and the first recorded human epizootic from a dog variant in the New World occurred in 1776 in the Greater Antilles. After that the frequency of epizootics increased and spillover into American mesocarnivores occurred (15).

The prevalence of RABV in bat species is variable. In big brown bats (*Eptesicus fuscus*), the most frequently submitted animal for RABV diagnostics in the United States, seroprevalence ranges from 3% to 35% in parts of the USA and Canada, while the actual virus is detected in 1% to 6% of big brown bats (30). An experimental inoculation of big brown bats with RABV resulted in 40% mortality, 6% of

which seroconverted. This experiment recorded an overall 35% seroconversion rate of 43 inoculated bats. Humoral immunity did not persist and only three bats were seropositive 175 days post inoculation (DPI). Bats were then inoculated a second time and again had a 40% mortality rate and 60% seroconverted. Thirty-three percent of bats that died due to RABV infection seroconverted. When surviving bats were inoculated a third time mortality dropped to 6%, and 27% of the bats seroconverted (30). These data demonstrate that RABV does not have as high a mortality in bats compared to other mammals, and that seroconversion may not be protective. It also indicates the bat innate immune response may provide substantial protection to disease and mortality caused by RABV, as surviving bats did not always seroconvert.

A bat-associated RABV inoculated into grey-headed flying foxes (*Pteropus poliocephalus*) had similar results. This experimental design was unique in that grey-headed flying foxes live in Australia where lyssaviruses have been identified, but RABV is not present. Two out of four inoculated bats succumbed to disease and were euthanized. Additionally, ten bats were inoculated with ABL, a lyssavirus isolated from a grey-headed flying fox, and three fell ill and were euthanized. Clinical disease and subsequent histopathology consistent with meningoencephalitis and nonsuppurative ganglioneuritis was present for both lyssaviruses. Of the bats euthanized none seroconverted. Five of the seven bats that did not demonstrate disease in response to ABL infection seroconverted. One of two RABVs inoculated bats seroconverted 70 DPI (31). These data further support the innate immune system as a robust means of defense against RABV in some cases. The similar disease pathology and results in both Yinpterochiroptera and Yangochiroptera may highlight the conservation of viral kinetics across lyssavirus species and conservation of viral-host ecology.

Lyssaviruses challenge the way in which the term “reservoir host” is interpreted. “Reservoir host” is generally defined as a host that maintains a virus in nature with minimal pathology and disease (32). Bats seem to be a reservoir for RABV and the origins of lyssaviruses, yet may succumb to rabies

disease (15, 30, 31). Disease and pathology is variable in bats dependent on the pathogenicity of the strain. While bats may be susceptible to rabies disease they are definitely unique in that in other species RABV are considered to have a morality rate of close to 100% (27, 30).

1.2.2: *Filoviridae*

Family *Filoviridae* is comprised of three genera, *Ebolavirus, Marburgvirus*, and *Cuevavirus* (26). The viruses are enveloped and filamentous in shape, with a single-stranded, negative sense RNA genome (33). *Cuevavirus* consists of a single species, Lloviu virus that was discovered in Spain in Schreiber's long-fingered fruit bats (*Miniopterus schreibersii*) in 2002. Its genome shares a 73.7% nucleotide homology to Zaire ebolavirus, suggesting a distant relation to the other filoviruses and has not been known to cause human disease (34). *Ebolavirus* and *Marburgvirus* cause a hemorrhagic fever in humans and non-human primates with a potential for mortality upwards of 90% dependent on strain (16, 35). Marburg virus (MARV) was first discovered in 1967 when two outbreaks in humans occurred simultaneously in Yugoslavia and Germany. It was determined that the virus spilled over from African green monkeys (*Chlorocebus tantalus*) imported from Uganda for research. Subsequent outbreaks in Africa were sporadic with many linked to caves and/or bats. In the mining village of Durba in northeastern Democratic Republic of the Congo (DRC) outbreaks of hemorrhagic fever disease have been associated with the Goroumbwa Mine since 1987 (16).

After a MARV outbreak persisted from 1998 through 2000 and infected 154 people with an 83% fatality rate, vertebrate and arthropod specimens were collected from Goroumbwa Mine to hone in on a wildlife reservoir. Although not published until 2007, specimens were collected in 1999 and represented eight species of bats, seven species of rodents, and a handful of shrews, crabs, a frog and more than 2,000 insects including crickets, spiders, wasps, moths, flies, streblids, nycteribiids, and mites. A MARV ELISA uncovered one species of insectivorous bat with antibodies to MARV; the eloquent horseshoe bat (*Rhinolophus eloquens*), and one species of fruit bat; the Egyptian fruit bat (*Rousettus aegyptiacus*).

ELISA results was supported by viral nucleic acid detected by PCR from both species of bats, as well as a second insectivorous species; the greater long-fingered bat (17). In 2005, PCR and antibody evidence was published that inferred bats as a natural reservoir for Ebola virus (36). Given the association of MARV with caves, and its close relation to Ebola virus, bats became the focal point of investigation as a potential wildlife reservoir for MARV (16).

To date, the largest MARV outbreak occurred in Angola in 2005 infecting 374 people with an 88% fatality rate. The viral source was never identified, but an investigation of bats in nearby Gabon and the DRC found both antibody and nucleic acid evidence for MARV infection only in Egyptian fruit bats out of ten different species tested (19). It is noteworthy that neither *Miniopterus* nor *Rhinolophus* species were assayed in this investigation. In 2007 in Uganda, four people succumbed to hemorrhagic disease caused by MARV. Exposure was linked to the Kitaka Mine, which is the home to an estimated 100,000 Egyptian fruit bats. Egyptian fruit bats and bats from the *Hipposideros* genus were tested for MARV. Samples were collected both in late summer in 2007, and spring in 2008. Of the 611 Egyptian fruit bats tested in 2007, viral RNA was detected in 33 by PCR, and five strains of infectious MARV were isolated. These were the first infectious isolates obtained from a suspected reservoir species. All *Hipposideros* species had negative assay results (18).

Further support of bats as the reservoir for MARV, and elucidation of viral ecology was found in Python Cave in Queen Elizabeth National Park. Python Cave is home to more than 40,000 Egyptian fruit bats, and it seems no other bat species reside there. Two independent cases of Marburg hemorrhagic fever in tourists were connected to this cave in 2007 and 2008. Bat samples were collected in 2008 and 2009, during breeding and birthing seasons. Of the 1622 bats tested, viral RNA was detected in 40 by PCR, seven new isolates were obtained, and 250 bats were seropositive. There was no evidence of vertical transmission. Samples collected during a period when juvenile bats were aged at six months, and fully weaned, had a greater prevalence of viral RNA detected in tissues by PCR compared to adults; 12%

versus 4.2%, respectively. During a different period, when juveniles were aged at three months, and recently weaned but still had maternal antibodies, the prevalence of viral RNA detected in tissues by PCR was 1.7% in young juveniles compared to 5.7% in adults. Based on these data it was concluded that Marburg virus is maintained by older juvenile bats that are independent of their mothers and no longer have maternal antibodies to confer protection (16).

While epidemiological data and laboratory data identify Egyptian fruit bats as a reservoir for MARV, experimental infections assist to ascertain mode of transmission and provide controlled data to further support Egyptian fruit bats as a reservoir. An experimental infection of five-month old Egyptian fruit bats used a second passage strain of Marburg virus isolated from a naturally infected Egyptian fruit bat. All animals in this experiment did not show overt signs of disease, and maintained a healthy body weight and body temperature. Viremia was detected from day one to day ten post inoculation, and seroconversion occurred after day ten. In addition to viremia, the virus was found to have tropism for predominately spleen, liver and skin from the inoculation site through day ten. However, multiple tissue types, including but not limited to, heart, kidney, adrenal gland, lung, intestine, testes, salivary gland, brain, and bladder had viral RNA present by qRT-PCR in at least one animal, suggesting, in some cases, a broad tissue predilection is possible. Through the progression of the study, viral RNA presence in tissues waned, but the spleen remained positive, albeit weak, through day 28 post inoculation. Of particular interest were those organs that might facilitate viral shedding: large intestine, bladder, kidney and salivary gland. Of the oral swabs collected, viral RNA was detected on days four thru 14, offering one possible route of transmission, and viral RNA was identified in rectal swabs collected on days four thru 11 offering a second route of transmission (37). Experimental transmission of infected bats to naïve bats has yet to be demonstrated.

The epidemiology, serology, genomic material found in numerous tissues, isolation of the virus in wild-caught bats, and experimental infection that demonstrates susceptibility and minimal pathology all

provide strong evidence that bats, specifically Egyptian fruit bats, are a reservoir for MARV (16-19). Given that some of the first evidence to support bats as a reservoir of MARV was found in a wild-caught eloquent horseshoe bat and a greater long-fingered bat in addition to Egyptian fruit bats (16), but has not been further explored, and in combination with studies that indicate sympatry—the inter-mingling of species—is a major trait associated with viral richness in bats (38, 39), field investigations in additional bat species may be warranted.

Ebola virus was first documented in 1976 when two near over-lapping out breaks occurred in the DRC and Sudan that, respectively, had 318 and 284 cases both with 79% mortality. The two outbreaks were caused by two different strains of virus, and given the ~2000 km that separate the countries, it was concluded that the sources for each outbreak were local and independent of each other (40). In the aftermath of the DRC outbreak, more than 800 bedbugs and 147 mammals, comprised predominately of rodents, were collected in attempt to find the viral source with no success (41). The Sudan outbreak was suspected to be caused by exposure to a mammal in a cotton factory but none of the 501 shrews, bats, rodents, a single toad, and lizards collected from this site had evidence for Ebola virus infection (42). Another investigation in the DRC and Cameroon collected samples in 1979 and 1980 from 117 species consisting of predominately rodents and bats, and yielded a single positive serum sample from a flying squirrel (*Anomalurus derbianus*) by indirect fluorescent antibody assay, but not by a confirmatory radioimmunoassay. All tissues were negative (40).

The sporadic nature of Ebola virus outbreaks, coupled with temporal distance between outbreaks hampers field investigations to determine the reservoir (43). While bats have been incriminated as a reservoir for Ebola virus since the initial documented outbreaks, it was not until 2005 that bats became the sole suspect. Between 2001 and 2005 outbreaks of Ebola virus in humans were linked to outbreaks in gorillas and chimpanzees. In 2003 investigations of the viral source for the great ape epidemics occurred, with the goal of connecting these non-human primate outbreaks to human outbreaks. Over 1,000 animals

were captured including bats, birds and small terrestrial vertebrates. The bats were the only animals with evidence for Ebola virus infection, and this was the first study with positive diagnostics for Ebola virus in bats. Four out of 17 hammer-headed bats (*Hypsignathus monstrosus*), eight out of 117 Franquet's epauletted fruit bat (*Epomops franqueti*), and four out of 58 little collared fruit bats (*Myonycteris torquata*) were IgG seropositive for Ebola virus. Additionally, four out of 21, five out of 117, and four out of 141 of the same bat species, respectively, had viral RNA detected by PCR in spleens and liver; tissues with which Ebola virus has tropism. Bats that had viral RNA in tissues were seronegative (36). Five months later repeat diagnostics from one of the collection locations demonstrated a drop in the detection of viral RNA by PCR from 22.6% to 2.2% and an increase in animals that seroconverted from 0% to 7.5% (36, 44). This demonstrated that animals cleared virus, and an adaptive immune response may be protective, highlighting the elusiveness of the viral ecology.

Multiple serosurveys were conducted in bats to provide evidence of the susceptibility of bats to Ebola virus. In the Republic of the Congo (RC), Gabon, and Senegal, 2070 bat samples were collected between 2003 and 2006. No bat samples collected from Senegal were seropositive. In RC and Gabon, across Ebola virus epidemic and non-epidemic areas, there was a 5% seroprevalence in Franquet's epauletted fruit bat, hammer-headed bats, and little collared fruit bats (45). Between December 2006 and December 2013, 748 serum samples from straw-colored fruit bats (*Eidolon helvum*) in Zambia, a country surrounded by those that had epidemics of Ebola virus but thus far, itself, has not had the misfortune, were assayed by ELISA with 8.6% prevalence. RT-PCR was performed on tissue samples and no viral RNA was detected (46). Straw colored fruit bats have been documented to be both non-migratory and migratory and travel up to ~2000 km (47). This may explain how seropositive bats were identified in a country that had no documented Ebola virus cases. Between May and June 2007 in Ghana, a study focused on non-migratory species of bats. Out of 88 serum samples, 32 were positive for IgG by ELISA including Franquet's epauletted fruit bat, Gambian epauletted fruit bat (*Epomops gambianus*), hammer-headed bats, and Veldkamp's bat (*Nanonycteris veldkampii*). A single Buettikofer's epauletted fruit bat

(*Epomops buettikoferi*) was tested and was negative. Seven ELISA positive sera were also positive by western blot (48). Again, in Ghana between 2008 and 2009, 262 samples from straw-colored fruit bats and hammer headed bats were collected. One straw-colored fruit bat had an IgG antibody titer of 80 to Ebola virus via indirect fluorescent antibody assay. Results were confirmed with western blot. Three hammer-headed bats were assayed and negative (49).

The first experimental inoculation of bats with Ebola virus occurred in 1996 in which wild-caught Angolan free-tailed bats (*Tadarida condylurus*), little free-tailed bats (*Tadarida pumilus*) and Wahlberg's epauletted fruit bats (*Epomorphorus wahlbergi*) were inoculated subcutaneously. In addition to these three bat species, pigeons, numerous amphibian species, a variety of insect species, and 28 different plant species were infected with Zaire ebolavirus (EBOV). While a brown house snake (*Lamprophis fuliginosus*), a mouse (*Mus musculus*), and a social spider (*Stegodyphus dumicola*) had positive tissue samples post inoculation with low titers (FFU/ml), it was the three bat species that had virus detected most consistently with titers of 10^2 to 10^7 FFU/ml. All other specimens were negative (43).

A second inoculation experiment inoculated Egyptian fruit bats with EBOV, Sudan (SUDV), Bundibugyo (BDBV), Tai Forest (TAFV), and Reston (RESTV) Ebolavirus species. No bats showed any signs of disease. SUDV RNA was detected in skin from the inoculation site, liver, spleen, axillary lymph node, and urinary bladder at 5 and 10 DPI. EBOV, BDBV, and RESTV RNA was detected in skin from the inoculation site at 5 and 10 DPI, and axillary lymph nodes at 10 DPI. TAFV was detected only in skin of the inoculation site at 5 and 10 DPI. No virus was detected in oral and rectal swabs and no remarkable histopathological lesions was observed. This same investigation executed inoculations side-by-side with MARV. Comparatively, MARV had a much broader tissue tropism, bats were viremic, had foci of cellular infiltrates in the liver as determined by histology, as well as antigen staining in these foci (50). Results for Ebola virus were recapitulated in another study where EBOV was inoculated into Egyptian fruit bats, and naïve Egyptian fruit bats were exposed to inoculated bats to study transmission.

The naïve bats did not seroconvert, had no evidence of infection, and were subsequently inoculated directly. Bats seroconverted with titers greatest at 28 DPI but beginning to wane by 37 DPI. Ebola virus RNA was detected in various tissues at different time points, including stomach of intraperitoneal inoculated bats, skin, liver, lung, and spleen. Virus was not isolated from any tissue. All oral, nasal, vaginal, penile and rectal swabs were negative (51).

The straw colored fruit bat, Gambian epauletted fruit bat, Egyptian fruit bat, Franquet's epauletted fruit bat and hammer-headed bats have all been found to be seropositive albeit a low prevalence (45, 46, 48, 49). Wild-caught hammer-headed bats, Franquet's epauletted fruit bats, and little collared fruit bats have all had viral RNA detected in tissues (36). Given that no other wild-caught animal species have demonstrated robust evidence of Ebola virus infection, and that ample evidence exists to support bats as the reservoir for the related MARV, bats are the dominant reservoir suspects. However, viral RNA has not been detected in bats since the initial investigation and Ebola virus has never been isolated from a bat (44). Furthermore, inoculation experiments demonstrate a different pathophysiology and infection time course which serves as a reminder that Ebola viruses and MARV are not synonymous (51). Ebola virus data may suggest that the route of infection in bats is different from MARV, or the viral ecology is unique, or it might suggest that bats are susceptible to Ebola virus but serve as an incidental host and not the reservoir.

1.2.3: *Henipavirus*

The genera *Henipavirus* has five species, two of which have significant public health implications, Hendra virus and Nipah virus (26). Henipaviruses are pleomorphic, enveloped viruses with a non-segmented, negative sense, single stranded RNA genome (27, 52).

Hendra virus (HeV) was first recognized in 1994 when two outbreaks occurred in two different locations in Queensland, Australia in which both humans and horses suffered from acute respiratory

disease and/or encephalitis (53, 54). It was hypothesized the reservoir host for the virus was a bat based on a paper published in 1974 suggesting bats were associated with multiple viruses and may be ample reservoir hosts (55), and two publications that isolated a paramyxovirus from bats—one in India (56) and one in Brazil (57). This hypothesis was supported with serological data from more than 5000 samples taken from 46 different animal species. The only animals that were seropositive were pteropid bats with a 4% prevalence in four different species; the grey-headed flying fox (*Pteropus poliocephalus*), the black flying fox (*Pteropus alecto*), the little red flying fox (*Pteropus scapulatus*), and the spectacled flying-fox (*Pteropus conspicillatus*) (58). Serosurveys conducted in humans yielded no seroconversion despite some of the participants having been exposed to bats (59, 60). This suggested that transmission was by a novel route, and given that the HeV outbreaks in horses occurred during the breeding season of three out of the four bat species that were seropositive, birthing fluids and fetuses became the focus for finding the virus (60). In 1996, HeV was isolated from the uterine fluid of one bat and fetuses of two bats out of 465 pteropid samples assayed via a RK13 cell culture system. Cell culture results were confirmed with qRT-PCR, sequencing, indirect fluorescent antibody assay, and electron microscopy (60).

Experimental inoculation studies of HeV in grey-headed fruit bats (*Pteropus poliocephalus*) resulted in seroconversion at three weeks post inoculation. There were no signs of clinical disease and no pathology on necropsy. Two bats with the highest antibody titers showed vasculitis on histology, predominately in mesenteric and gastrointestinal arteries with HeV antigen present in these lesions on immunohistochemistry (IHC). Virus was not isolated from any tissue of any bat, and urine and feces were negative. In the same study, naïve bats and horses were exposed to inoculated bats and neither experimental group seroconverted. Horses were then inoculated with HeV, and with the exception of one horse that had no apparent clinical disease; inoculated horses developed varying degrees of pyrexia, respiratory disease, and lethargy. Virus was not isolated from the horse that was asymptomatic. Symptomatic horses had vasculitis and interstitial pneumonia, and one had lymphocytic meningitis. Virus was not detected in nasal or rectal swabs nor urine but was isolated from kidneys and urine

extracted from the bladder. Given that field isolation of HeV occurred in pregnant bats, it is not surprising that natural transmission from non-pregnant bats to horses was not achieved (61). Another experimental inoculation study conducted on pregnant grey-headed fruit bats yielded no signs of clinical disease and no teratogenic effect in the fetuses. Inoculated bats seroconverted as determined by ELISA and serum neutralization assay. None of the fetuses seroconverted. HeV was isolated from the heart and buffy coat of one bat, and spleen and kidney of its fetus. The virus was also isolated from the kidney, heat, and spleen from a second bat but not from that bat's fetus. An additional two bats had no virus isolated from any tissue. No gross pathology was seen, and histopathology observed in vasculature was described as fibrinoid degeneration, perivascular cuffing by mononuclear cells, and pyknotic endothelium with antigen present as determined by IHC. In a single bat, there was immunostaining of the placental veins but all fetuses were negative for viral antigen by IHC. This corroborates field studies and gives evidence to vertical inter-species transmission. It does not provide evidence as to how spillover to other species may occur, but urogenital swabs were not taken nor did pregnancies go to term to assess viral load in birthing fluids (62).

While mode of spillover of HeV has not been fully elucidated, it seems to be associated with parturition. Epidemiology, seropositive wild bats, isolation of the virus from pteropid species, lack of evidence of virus in other animal species aside from humans and horses, and experimental inoculations that demonstrate no clinical disease in bats all support pteropid bat species as a reservoir for HeV.

Nipah virus (NiV) was first detected in Singapore and Malaysia in 1999 after an outbreak that began in 1998 resulted in 105 human deaths due to encephalitis. A concurrent respiratory disease outbreak in pigs resulted in more than one million animals culled. Initially the etiological agent for the outbreak was thought to be Japanese encephalitis virus, but when pigs were shipped from one part of Malaysia to another and outbreaks followed in pig abattoir workers, it was identified that transmission occurred through direct contact rather than vectored by a mosquito (63). Given the structural, serologic,

antigenic and molecular commonalities with HeV, bats were the primary reservoir suspects. Sera from multiple genera of bats, as well as wild boar, domestic dogs and peridomestic animals were tested for antibodies to NiV. All non-bat species tested negative by indirect ELISA. Five species of bats were antibody positive; 31% of island flying foxes (*Pteropus hypomelanus*), 17% of Malayan flying foxes (*Pteropus vampyrus*), 5% of cave bats (*Eonycteris spelaea*), 4% of lesser dog-faced fruit bats (*Cynopterus brachyotis*), and 3% of house bats (*Scotophilus kuhli*). The virus was isolated from the urine of island flying foxes by lying tarps under roosts to collect pooled urine. From three field excursions made to collect urine, the final one resulted in the isolation of NiV on Vero cells detected by novel CPE, indirect fluorescent antibody assay, and confirmed with sequencing (64).

In the original NiV outbreaks, pigs served as an amplifying host; however, in the years to follow more outbreaks occurred in people sans pigs. In Bangladesh between 2001 and 2007, there were a total of 122 human NiV cases with a 71% fatality. Based on heterogeneity of NiV strains it was purported that 23 different introductions of the virus were made from Indian flying foxes (*Pteropus giganteus*)—the only fruit bat species to inhabit Bangladesh. Once introduced into human populations, human-to-human transmission was possible, different from the Malaysian outbreaks in which human-to-human transmission could not be confirmed (65). In a 2005 outbreak, it was discovered that there was a connection between human infection and fresh date palm sap. Date palm sap is collected by strapping clay pots to trees (*Phoenix sylvestris*), stripping the bark near the top of the pot, leaving the pot overnight, and allowing sap to seep into the pot for collection the following morning. Bats were frequently heard in the trees at night, feces were commonly seen on the clay pots after collection, and occasionally a dead bat was seen in the pots. It was determined that the sap was contaminated with virus and many infections were food-borne (66).

When captive-bred, grey-headed fruit bats are experimentally inoculated with NiV, animals appear clinically normal. Seventeen animals were inoculated, euthanized at predetermined time points,

and monitored for a total of 22 days. They were afebrile and had no weight loss. Virus was isolated from urine from a single male bat at 12, 16 and 18 DPI, and from kidney of a different male bat and uterus of a female bat at 7 DPI. On necropsy, organs appeared within normal limits and there were varying degrees of nephritis and hepatitis in multiple but not all bats (67).

The epidemiology, seroprevalence, isolation of virus, evidence to demonstrate route of transmission, and lack of NiV found in other animal species unquestionably support the hypothesis that bats are the reservoir for NiV. Experimental inoculation data of NiV in bats corroborates this hypothesis as NiV causes no apparent disease and has minimal pathology, in line with what is expected from a reservoir host.

1.2.4: *Coronavirinae*

Subfamily *Coronavirinae* consists of four genera: *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus* (26). Mammals are generally infected with alpha and beta viruses and birds with gamma and delta. There are six coronaviruses that are associated with human disease. Four of these are etiological agents for the common cold; alphacoronavirus HCoV-229E and HCoV-NL63, and betacoronaviruses HCoV-OC43 and HCoV-HKU1. Severe acute respiratory syndrome coronavirus and Middle East respiratory syndrome coronavirus make up the rest of the human coronaviruses and are novel in the severity of disease they incite. Coronaviruses are spherical, enveloped viruses with a positive-sense, single-stranded RNA genome (68).

Severe acute respiratory syndrome coronavirus (SARS-CoV) emerged in Guangdong Province, China in late 2002. Thereafter, reports of a severe febrile respiratory disease in humans quickly emerged from Vietnam, Canada, Hong Kong, and more than 20 different countries infecting more than 8000 people with more than 900 deaths (69). In 2003, the etiological agent for the disease was identified as a novel coronavirus. The severity of disease caused by a coronavirus was unique, as previously

coronaviruses were thought to predominately cause mild respiratory disease in humans (70, 71). Due to a high seropositivity in food handlers and people that worked in live-animal markets it was suspected that the original source of the virus was an animal (72). Investigations commenced that placed masked palm civets (*Pauma larvata*) at the forefront of possible reservoir species suspects (73). As studies progressed it was found that there was a seroprevalence disparity between civets on farms and civets in markets. Civets in the market had a 78% seroprevalence while those on farms had a prevalence of 40%. It was concluded that a percentage of civets were exposed to SARS-CoV at the market place, and therefore may not be the reservoir (74). When civets were experimentally infected with SARS-CoV they demonstrated clinical signs of disease including pyrexia and lethargy, lending evidence to the hypothesis that civets were not the reservoir but rather an amplifying host (75). With the attention bats were getting as reservoir species for other pathogens, and the increase in bats being sold in markets for food and traditional Chinese medicine, bats became the new suspect in the search for the reservoir for SARS-CoV. Four hundred and eight bats from nine different species were sampled in China. Three species from the genera *Rhinolophus* were seropositive to SARS-CoV with a prevalence of 28% to 71%, dependent on species. Fecal samples tested positive by qRT-PCR and a whole genome from feces was sequenced with 92% homogeneity to a human strain (76).

Middle East respiratory syndrome coronavirus (MERS-CoV) was first diagnosed in a Saudi man in 2012 that died of progressive respiratory and renal failure eleven days after admittance to the hospital (77). Currently, the World Health Organizations reports 2103 laboratory confirmed cases with more than 700 virus related deaths in 27 countries. Unlike SARS-CoV, where cases of viral infection subsided after the epidemic was brought under control, MERS-CoV lingers with sporadic cases, predominately in the Middle East. The most recent case occurred in November of 2017 (78). After the isolation of MERS-CoV from a human, investigation to identify the reservoir host quickly began. An array of farm animals were screened for antibodies to MERS-CoV, including, goats, sheep, cows, alpacas, Bactrian camels and dromedary camels. All of the dromedaries were seropositive, while 14% of the Bactrian camels were

seropositive, and none of the other species demonstrated seroconversion (79). With seroprevalence in dromedary camels upwards of 90% in certain regions of the Middle East, the isolation MERS-CoV from dromedary camels, susceptibility and minimal disease on experimental inoculations, and evidence for direct transmission from camels to humans; dromedary camels are considered a host for MERS-CoV (79-83). However, coronaviruses with high nucleotide identity to MERS-CoV were found in bats.

Lung and alimentary tissues from 5,481 bats from 21 different species were assayed via RT-PCR for coronavirus RNA in Japan. There was a 29% prevalence of Ty-BatCoVHKU4 virus in the gastrointestinal tract from the lesser bamboo bat (*Tylonycteris pachypus*), and 25% prevalence of Pi-BatCoVHKU5 in the gastrointestinal tract of Japanese pipistrelle (*Pipistrellus abramus*) with no signs of disease. Pi-BatCoVHKU5 shares 92.1% to 92.3% amino acid identity to MERS-CoV while Ty-BatCoVHKU4 shares 89.6% to 90% of the RNA dependent RNA polymerase gene (84). RNA dependent RNA polymerase (RdRp) genes are highly conserved (68). The more mutable S gene of Pi-BatCoVHKU5 had a 66.8% to 76.4% amino acid identity and Ty-BatCoVHKU4 had a 63.4% to 64.5% amino acid identity to MERS-CoV. Molecular clock analysis estimates the time of divergence between the three viruses to be hundreds of years ago (84).

Shortly after the discovery of Ty-BatCoVHKU4 and Pi-BatCoVHKU5 CoV, coronavirus RNA was discovered by PCR in 220 out of 732 fecal samples collected off tarps, and 7 out of 91 rectal swabs from seven different species of bats in Saudi Arabia at locations where people contracted MERS-CoV. Amongst these viral RNA positive samples was an amplified 190-nucleotide product for RdRp gene that had 100% nucleotide identity to the index case for MERS-CoV (85). These findings laid the groundwork for the hypothesis that MERS-CoV originated in bats and subsequently spilled over into camels and humans.

Across multiple locations in Ghana, West Africa, as well as Germany, the Netherlands, Romania, and Ukraine, 5030 fecal samples were collected from different bat species and screened for coronaviruses. Positive samples, by RT-PCR, came from 46 out of 185 specimens from Gambian slit-faced bats from Ghana (*Nycteris gambiensis*). The amplified fragment that included the RdRp gene from this novel betacoronavirus had a 90.4% to 91.2% amino acid similarity to Ty-BatCoVHKU4 and Pi-BatCoVHKU5 and a 92.5% amino acid similarity to MERS-CoV. Furthermore, positive samples were found in pipistrellis bats (*Pipistrellus pipistrellus*, *P. nathusii*, and *P. pygmaeus*) in the Netherlands, Romania, and Ukraine. Forty of 272 had coronavirus RNA detected by PCR in fecal samples with a 98.2% amino acid homology to MERS-CoV and 94.1% to 94.5% amino acid identity to Ty-BatCoVHKU4 and Pi-BatCoVHKU5. Again, the gene amplified was the highly conserved RdRp gene (86). Another study found RNA from a novel coronavirus with high similarity to MERS-CoV in five out of 62 fecal samples from Zulu pipistrelle bats (*Neoromicia zuluensis*) in South Africa. Four of the five positive samples were alphacoronaviruses and one was a betacoronavirus and was dubbed PML/2011. RT-PCR amplification of the RdRp gene for PML/2011 yielded an 816-nucleotide fragment that had a single amino acid difference from MERS-CoV. The virus differed from Ty-BatCoVHKU4 and Pi-BatCoVHKU5 by 1.8% at the amino acid level (87). In addition to these studies, betacoronaviruses have also been detected in a Savi's pipistrelle bat (*Hypsugo savii*) in Spain, bat guano in Thailand, and a *Nyxtinomops* bat species in Mexico (88-90). Given these data it appears as if betacoronaviruses have a diffuse geographical distribution.

The broad distribution of MERS-CoV-like viruses in bats is highly suggestive MERS-CoV originated in bats; however, it is not definitive. There is no clear epidemiology to suggest MERS-CoV originated in bats nor has the virus been isolated from bats. There has been one experimental inoculation of bats with MERS-CoV. Ten Jamaican fruit bats were inoculated intranasally and intraperitoneally with 10^5 $TCID_{50}$ MERS-CoV strain EMC/2012. All bats appeared clinically normal throughout the duration of the study. Viral RNA was detected in oral swabs and rectal swabs by RT-PCR for up to 9 DPI and suggested bats shed virus via both routes. Additionally, virus was detected in numerous tissues and

blood, predominately at time points prior to 14 DPI. Infectious virus was isolated from lungs, bladder and nasal turbinates at 2, 7 and 14 DPI. Histopathology was minimal and included multifocal interstitial pneumonia and mild rhinitis in two bats. A single bat seroconverted at 14 DPI. These data were consistent with what is expected from reservoir hosts—infection with minimal to no pathology; however, Jamaican fruit bats are a New World bat and so unlikely to be an original host (91). Furthermore, bat speciation occurred millions of years ago (4) and bat physiology is not well enough delineated to know if what happens in one species recapitulates what might happen in another.

The emergence of SARS-CoV and MERS-CoV spurred a frenzy of identifying bat coronaviruses, and postulations that bats are the original host of coronaviruses. Literature suggests that bats harbor more coronaviruses than any other animal species (21). In addition to the identification of SARS-CoV in bats and close relatives to MERS-CoV in bats, other coronaviruses that group closely with less pathogenic human coronaviruses have also been detected. HCoV-229E is an alphacoronavirus that causes mild respiratory disease in humans worldwide. A search for coronaviruses in straw-colored fruit bats, belonging to the *Hipposideridae* family, in Ghana was conducted by passively collecting fecal samples from a colony of 300,000 bats. Seven specimens had RNA for a novel coronavirus RdRp gene by RT-PCR. Amongst these novel coronavirus RdRp sequences, was an alphavirus with a 92% nucleotide identity of the RdRp gene with HCoV-229E and labeled bat 229E-related CoV lineage 1. The bat virus and the human virus were predicted to share a common ancestor 208 to 322 years ago (92). Another investigation of 2087 bats comprising 11 species in Ghana found 229E-related CoV RNA by RT-PCR in 81 fecal samples collected from Hipposideridae bats. The full-length virus was sequenced and compared to multiple strains of human coronavirus HCoV-229E, SARS-CoV and related viruses, and animal coronaviruses via Bayesian phylogenetic analysis. Bat 229E-related CoV lineage 1 and an alpaca coronavirus formed one species in the analysis. The alpaca virus took an intermediate position between HCoV-229E and bat 229E-related CoV lineage 1. It was suggested that HCoV-229E may have been a bat

virus that spilled into alpacas and then into humans—further implying that bats may be a reservoir for coronaviruses (93).

1.2.5: *Hepacivirus* and *Pegivirus*

Not only do bats harbor multiple viruses with significant public health consequences, they also may be the original hosts for family *Paramyxoviridae*, subfamily *Coronavirinae* and genera *Lyssavirus* (as discussed) (20), *Hepacivirus,* and *Pegivirus* (23). *Hepacivirus* and *Pegivirus* belong to family *Flaviviridae*, a group of enveloped, spherical or icosahedral viruses with non-segmented, single-stranded, positive-sense RNA genomes. *Hepacivirus* species include hepatitis C virus (HCV), GB virus B (GBV-B), and non-human primate hepaciviruses (NPHV). HCV infects approximately ~3% of the human population causing hepatic fibrosis and cirrhosis, and inducing liver neoplasia. *Pegiviruses* include GBV-A, GBV-C and GBV-D. GBV-A and GBV-C have tropism for the liver in humans and NHP. GBV-A was discovered in humans but it was most likely incidental, and rather NHPs are the native host. GBV-C has been found in up to 15% of human populations as well as chimpanzees, but does not appear to cause disease. GBV-D was identified in a fruit bat in Bangladesh (94). A survey sampled 40 different bats species for a total of 1581 bat sera and 83 tissue samples from eight different countries and revealed a broad geographical and phylogenetic flavivirus distribution. Seventy-eight sera samples, one lung, and one rectal swab had flavivirus RNA detected by deep sequencing and qRT-PCR, with a 24% to 100% amino acid homology to *Hepacivirus* or *Pegivirus*. Phylogenetic analysis that included previously described non-bat viruses (HCV, NPHV, GBV-B, GBV-A, GBV-C, GBV-D) showed that all the previously described non-bat viruses clustered within the bat viruses. Given the global distribution and range of bat species that were positive, in combination with phylogeny of all known *Hepaciviruses* and *Pegiviruses*, bats may be a major reservoir for these viruses (23).

1.2.6: *Paramyxoviridae*

Paramyxoviridae contains numerous genera of viruses that infect humans and animals including measles, distemper, mumps, parainfluenza, Newcastle disease, and henipaviruses. An extensive Bayesian analysis of 51 species of paramyxoviruses from six genera demonstrated that a salmon paramyxovirus clustered with a rodentian-morbilli-henipavirus and human respirovirus clade, generating the conclusion that morbilliviruses may have originated in rodents. Yet, the study did not exclude bats as an origin species based on a lack of paramyxovirus data in bats at the time (22). A survey for paramyxoviruses was conducted in 86 bat species constituting 4,954 individuals and 33 rodent species with 4,324 individuals in 15 different countries. RT-PCR and deep sequencing yielded a 3.3% and 3.1% prevalence in bats and rodent, respectively. A total of 66 novel paramyxoviruses were discovered. Of particular interest was the discovery of a bat *Rubulavirus* with 89.5% to 90% amino acid identity to mumps virus, a highly contagious human-to-human spread pathogen with significant public health consequences. Additionally, viral fragments and one full genome sequence were found in a straw-colored fruit bat in Ghana, Africa with high homology to the henipaviruses, suggesting that the ancestral virus to Hendra and Nipah virus came from Africa. While virus prevalence was similar between the two animal orders, rodent viruses had low divergence and clustered tightly within three clades while bat viruses had a broad distribution across the phylogenetic tree and high divergence, this may indicate that bats are a major source for paramyxoviruses. In order to determine which mammalian host order had the most robust spillover events, host-traits were analyzed in concordance with a viral phylogenetic tree. This analysis revealed that host switches from bats into mice, carnivores, primates, and cetartiodactyls occurred with greater frequency than spillover from mice, carnivores, primates, and cetartiodactyls into bats, mice, carnivores, primates, and cetartiodactyls. These data indicate that bats are a major reservoir for paramyxoviruses (20).

1.2.7: *Orthohepadnavirus*

Hepadnaviridae is a family of double-stranded DNA viruses comprised of two genera: those that infect mammals, *Orthohepadnavirus*, and those that infect birds, *Avihepadnavirus*. The most well characterized hepadnavirus is hepatitis B virus (HBV), which is estimated to infect more than 325 million people globally (95). Phylogenetic studies date the human-HBV relationship back at least 15,000 years; however, *Avihepadnaviruses* date back 19 Ma, suggesting that HBV may have much older origins that have not been uncovered (24).

Serum and liver from 3080 bats, representing 54 different species, from Panama, Brazil, Gabon, Ghana, Germany, Papua New Guinea and Australia were assayed via PCR for hepadnaviruses. Ten samples from three different bat species of both New World and Old World distribution had RNA present, comprising three novel strains. On histology, minimal cellular infiltrates in the liver were described. The bat hepadnaviruses form two monophyletic clades, clustering according to geographic distribution within New World human genotypes, New World non-human primate and New World rodent hepadnaviruses according to Bayesian phylogenetic analysis. Furthermore one of the three strains of bat hepadnavirus was able to infect and replicate in human hepatoma HepG2 cells, possibly hinting at zoonotic potential (24). Because of these data it is suggested that bats may be an origin for HBV and other mammalian hepadnaviruses (24, 25). There are multiple other hypothesis for the origins of HBV including parallel evolution between humans and non-human primates, a common viral ancestor infected anatomically modern humans and human migration out of Africa disseminated the virus globally, and cross-species transmission between human HBV and non-human primate HBV occurred (25). More research needs to be conducted in order to support or reject any of the hypothesis, including the bat hypothesis.

1.3: Traits that Make Bats Unusual

As described, bats maintain many high-profile viruses that cause substantial disease and mortality in humans. This drives bat research in an attempt to understand the mechanism by which bats can host such a great variety viruses (96). It is true rodents are also considered significant reservoirs for zoonotic viruses, yet bats harbor significantly more zoonotic viruses than rodents (38). Numerous hypothesis have been proposed to explain the mechanism, including the age of order Chiroptera and potential for co-evolution with numerous viruses, their immunophysiology facilitating infection and spread with minimal disease, they are the only mammal capable of sustained flight and this feature may result in distinct molecular physiology, their life span is longer than what would be expected given their metabolic rate to body weight ratio, they are gregarious and may roost in great numbers often with multiple species (97-99).

1.3.1: The Ancient Age of Bats and Viral Co-Evolution

Does the ancient age of bats facilitate the success of bats as a reservoir for viruses? Molecular dating sets the age of Chiroptera between 58.9 and 88 Ma and deduces that all 18 extant families existed 33.9 Ma (4-6). Order Rodentia is another major reservoir for human pathogens and is estimated to have diversified 64 to 88 Ma (100, 101). This order contains more species than any other, with more than 2,000 documented—twice as many as bats. Like bats, rodents inhabit every continent except Antarctica. Molecular dating suggests the 34 extant families of rodents were established 22 Ma, during the Neogene (101). Modern birds are estimated to have split into modern orders 70 to 120 Ma based on molecular dating, suggesting the modern orders pre-date and survived the K-Pg boundary event (2, 102). Within the avian class there are 23 orders. Amongst these orders are the Anseriformes, waterfowl, which are the reservoir for avian influenza viruses (103). Waterfowl branched from the crown-group approximately 80 Ma (102). The order of rodents, bats, and waterfowl are all quite old and so millions of years of co-evolution with their viruses may explain the commensal relationship.

Order Carnivora arguably contains some of the most charismatic mammalian animals within its more than 280 recognized species comprised of 11 families. Molecular clock analysis date Carnivora to 55 Ma. Families diverged from crown-groups over a great expanse of time, for example 7.2 Ma for Hyaenidae (hyenas) and 28.6 Ma for Viverridae (civets) (104). Order Primates, which includes *Homo sapiens*, contains some discrepancy in the dates it emerged with molecular clock analysis targeting the origins between 55 and 95 Ma, and the oldest fossil dated 54 million years old (105, 106). Furthermore molecular clock analysis further dates superorder Xenarthra (sloths, armadillos, and anteaters), order Pholidota (pangolins), and order Proboscidea (elephants) at ~60 Ma (107). None of these orders are incriminated as reservoir hosts, yet are similarly aged to Chiroptera. Order Eulipotyphla contains moles, shrews, hedgehogs, amongst others, and is older than both Rodentia and Chiroptera at 71 to 86 Ma (100), and are not documented to harbor any zoonotic viruses.

Age of an order may facilitate the amount of time an animal has to co-evolve with its virome but does not indicate an inherent risk for spillover events. More aptly it suggests that cross-species transmission had the potential to occur for millions of years and may have (108). It does not explain why viral spillover from bats is currently observed with greater frequency than ever before. If age of an order were an indicator of how many viruses that order could maintain, then it might be expected to see a more homogenous viral spread across orders of similar or older age.

1.3.2: Bat Immunology

Given that bats host numerous viruses, yet seem to have minimal pathology, it has been suggested that bats have evolved immune mechanisms to facilitate a commensal relationship. As technology advances and more tools become available, molecular investigation becomes more accessible (96).

RNA isolated from the thymus of a juvenile male flying fox, and RNA isolated from a pool of mitogen-stimulated spleen, leukocytes, lymph node, unstimulated thymus and bone marrow was

stimulated with LPS and Ionomycin, sequenced, and transcriptomes acquired. Seventy genes were found to be involved in toll-like receptor cascades, 50 in B-cell activation, 79 in T-cell activation, 72 in natural killer (NK) cell cytotoxicity, and 41 in antigen presentation, comprising 3.5% of the total bat transcribed genes. Ultimately, the transcriptome analysis demonstrated that the overt flying fox immune system has the same parts-list as other mammals (109), although does not necessarily elicit functional differences. On a transcriptome level, any differences that exist between bat immune systems and other mammals to facilitate co-existence with zoonotic viruses is in the details.

1.3.2.1: Innate Immunity

The interferon family of genes, having evolved 250 Ma, is present even in the earliest jawed vertebrates—Gnathostomes; as well as cartilaginous and bony fish, amphibians, reptiles, birds and mammals (110, 111). There are three types of interferon (IFN) in mammals labeled types I, II and III. Type I and III IFN are the predominate responders to viral infection. IFN-α, -β, -ω, -ε, -ζ, -κ, τ, and δ constitute the type I IFNs with IFN-α and IFN-β the bulk of the interferon response (112). The black flying fox has ten IFN genes: three *IFN-α*, one *IFN-β*, one *IFN-ε*, and five *IFN-ω* that span a total of 250 kilobases (kb), a remarkably small size compared to other species. The type I IFN locus has a tendency to increase in association with evolutionary complexity, for example fish loci span a distance of ~25 kb, 350 kb in mice and 1,000 kb in pigs. The bat genome is contracted at 2.0 gigabases (Gb) and so a smaller locus may be expected, which is in line with birds and consistent with evidence that flying animals have smaller genomes (113, 114).

Interferon is generally unmeasurable in a healthy, uninfected system and increases within hours in response to infection. Black flying fox IFN-α is uniquely, constitutively expressed in twelve tissue-types assayed from three wild-caught, apparently healthy animals. IFN-β seems to only be constitutively expressed in the testes. Similar results were seen in lesser short-nosed fruit bats (*Cynopterus brachyotis*) (115) and Jamaican fruit bats (116). *In vitro* investigation recapitulates this data. Unstimulated primary

cell lines made from black flying fox tissues constitutively express IFN-α. Levels remain similar even after stimulation with polyI:C. In contrast, IFN-β production increases after cell stimulation with poly(I:C). The implications for having interferon that is always expressed are substantial and may be a means to immediately control viral replication upon infection, minimizing replication (115).

After the production of type I IFNs, IFN-α and IFN-β induce the Janus kinase/signal transducer (Jak/STAT) signaling cascade. STAT1 and STAT2 are components of this cascade and are present at basal levels in the cytoplasm in an unphosphorylated form, prior to stimulation. Upon stimulation, STAT1 and STAT2 are phosphorylated and form heterodimers with IFN regulatory factor-9 (IRF-9) that then translocate to the nucleus, resulting in transcription of interferon stimulated genes (ISG). This cascade of events further induces the body to take an anti-viral posture against invaders. In order to elucidate the conservation of this system compared to other mammals, STAT1 from primary kidney cells generated from a mature female Egyptian fruit bat was cloned and sequenced. The amino acid similarity between Egyptian fruit bat STAT1 and STAT1 from other mammals was high, 97% with horses, 96% with cows and 93% with humans, mice and domestic dogs, highlighting the evolutionary conservation of the protein and suggesting that function is the same. Indeed, functional exploration revealed that STAT1 is phosphorylated and translocates to the nucleus after stimulation with Rabies virus or IFN-α. Furthermore basal levels of STAT1 are present in all tissues assayed, including brain, muscle, heart, lung, liver, kidney and spleen, but expression is variable depending on tissue type with the liver having the highest mRNA expression (117). These data demonstrate that STAT1 function is the same in bats as it is other animals and infers that the Jak/STAT pathway is also conserved. The yet unanswered follow-up question generated from these data is if type I IFNS are constitutively expressed, then what prevents ISGs from constant stimulation.

Unlike type I IFN, type III IFN expression in bats is similar to that of other mammals and is not constitutively expressed (118, 119). Type III interferon, IFN-λ, is encoded by five to six exons. Different

vertebrate classes have varying numbers of functional genes, for example: four in humans, two in mice, and one in zebrafish and chickens (120). As the proposed evolutionary original interferon and generally highly conserved across species, bat type III interferon follows expected patterns. The Malayan flying fox has three loci (IFN-λ1, IFN-λ2, IFN-λ3) with IFN-λ2 possibly being a pseudogene, and two have been found in the black flying fox (IFN-λ1 and IFN-λ2). Aside from a 4 kb intron in black flying foxes, compared to ~1.1 kb intron in other mammalian species, gene structure appears to be conserved. Given the small size of the bat genome, having such a large intron seems peculiar and the intron may have an undiscovered function. Because up-regulation of IFN-λ2 in bat cell lines occurs only after transfection of polyI:C, and not simply treatment with polyI:C, there is some question as to what pattern recognition receptors set the cascade of events into motion for type III interferon production, possible only cytosolic. Mice use both cytosolic and endosomal pattern recognition receptors. Additionally, IFN-λ1 concentration is 100 times greater than IFN-λ2 in polyI:C transfected PaLuT02 cells (immortalized black flying fox lung cells) and primary cell culture from multiple bat tissue types including lung, liver, heart, kidney, small intestine, brain, and salivary gland. This is different from mice that produce IFN-λ1 and IFN-λ2 in near equal amounts in response to a stimulant. Furthermore, IFN-λ1 expression is restricted to lung, liver, and heart primary cells compared to IFN-λ2. The type III IFNs were also produced later after cell treatment compared to type I IFNs, 0.5 hours compared to 1.5 hours. Type III IFNs are considered the evolutionary origin of the interferon family but through time type I IFNs may have taken center stage as the primary responder to viral infection, particularly in bat species that express type I IFN constitutively. Given the differences between type I and III IFN expression in bats, the two systems may work in tandem with one another. It is also postulated that because so many viruses have accessory proteins that dampen the type I IFN response, the type III IFN may serve as a backup when the type I IFN response is mitigated (119).

After the production of IFN-λ, IFN-λ interacts cellular receptors: IFN-λR1 and IL10R2. Binding of IFN-λ to these receptors results in production of interferon-stimulated genes (ISGs) and allows for

defense against viruses. IFN-λR1 has high ligand specificity for type III IFNs, and is expressed only on epithelial cells in humans and mice, with variable expression between organs of each species. IL10R2 is part of a receptor complex for multiple cytokines and, given this, has a more ubiquitous distribution. The black flying fox IFN-λR1 has a 66 to 79% nucleotide homology to other mammalian IFN-λR1 and a 50 to 65% amino acid identity. The shared identity of black flying fox IL10R2 to other mammals is 75 to 85% at the nucleotide level and 63 to 78% at the amino acid level. Both are expressed on multiple tissue types, and IFN-λR1 is present on epithelial cells, as well as a mixed population of immune cells, and stimulation with IFN-λ results in ISG induction. Bat IFN-λR1 and IL10R2 distribution and functionality are consistent with what is seen in both mice and humans, and given the nucleotide homology, this system seems to be conserved in the animal species assayed. The presence of IFN-λR1 on epithelial cells from a variety of tissues types and hematopoietic cells indicates bats are well poised to take on an antiviral state should infection arise (118).

1.3.2.2: Cell-Mediated Immunity

Major histocompatibility complex class I (MHC-I) is present on all nucleated cells and platelets and is a major defender against intracellular pathogens, such as viruses. The complex contains a peptide binding grove (PBG) that, canonically has a rigid α-helix and proline on either side that limits the size of the antigen it can fit to 8-11 amino acids. When MHC-I bands a peptide, it presents the peptide to cytotoxic t cells. If the t cell antigen receptor is a match, it will bind to the antigen-MHC-I complex with co-stimulation of CD8 and induce apoptosis. Characterization of the MHC-1 molecule in black flying foxes demonstrates that all of the ancestral conserved genes are present. The MHC-1 region in big brown bats is ~1.2 megabases (Mb), compared to humans where the region is ~1.7 Mb. Again, this is consistent with the comparatively small size of the bat genome compared to the average mammalian genome of ~3.5 Gb. 3D protein modeling conducted to elicit the conformation of the PBG. Bat PBG has proline on either side consistent with what is documented in other mammals, but the 3D model was less rigid and

contained coils and turns that might allow for the accommodation of larger peptides, possibly making endogenous antigen presentation more efficient (121).

1.3.2.3: Antibody-Mediated Immunity

Antibodies function to dispel of pathogens by binding to them and neutralizing them, agglutinating or precipitating antigen to make ease of phagocytosis, or to activate complement pathways. Placental mammals have five classes of antibodies: IgA, IgD, IgE, IgG and IgM. When an animal encounters a microbe, IgM is the first to be produced. Daughter B cells then undergo class switching to produce a different antibody class dependent upon the demands of the body. Each class of antibody has a distinct function. IgG is the dominant isotype in sera. IgA is the dominate isotype in mucosal surfaces. IgE is specialized to respond to allergens. IgD is mainly an antigen receptor on naïve B-cells (122).

In the quest to understand the adaptive immune system of bats, transcriptome investigations to characterize the antibody repertoire were implemented. Three bat species belonging to suborder Yangochiroptera were used: little brown bats, big brown bats, and Seba's short-nosed fruit bat, and a member from family Pteropodidae as a representative of the Yinpteropodidae superorder—the short-nosed fruit bat (*Cynopterus sphinx*). Results demonstrated that bats have all five canonical immunoglobulins. IgM and IgA appeared to be the most conserved in comparison to other eutherian mammals. IgD was present in the little and big brown bats but absent in Seba's short-nosed fruit bat and the short-nosed fruit bat. The IgD transcriptome in the brown bats demonstrated the least homology amongst mammals, and so it may have been missed in the other bat species. Although rabbits do not produce IgD, and so if it is not present in some bat families, it would not be completely novel. IgE lacked the N-glycosylation site of CH1 similar to opossum and platypus, but different from humans, mouse, cattle and pigs. Furthermore, the N-glycosylation motif was missing in CH2 in the four bat IgEs, compared to other mammals, and bats had one N-glycosylation site in the CH3 domain, compared to three in human IgE. Phylogenetically, IgM and IgD were most homologous to mammals belonging to the order

Carnivora. IgE shared homology with order Carnivora but also horses, humans and pigs. Variation was observed amongst IgG from the four bat species, and phylogenetics demonstrated that IgG developed after the speciation of bats ~58.9 to 64 Ma. The greatest variation of bat IgG subclasses were seen in the hinge region—which is generally the site of greatest diversity in other species (123). These results demonstrated that bats have an immunoglobulin repertoire consistent with other mammals, despite some differences.

To assess the antibody repertoire on a protein level an investigation used Protein G and Protein A affinity columns to isolate IgG from black flying fox serum and plasma. The IgG depleted serum and plasma was processed with immobilized anti-Fab-specific antibody to capture IgM and IgA. The results demonstrated bands for heavy and light chain IgM but not for IgA. IgA constitutes 15-50% of the immunoglobulins found in human serum, but may be trace in the serum of black flying foxes as LC-MS/MS can detect a small fraction. LC-MS/MS can detect IgA in lavages from bat small and large intestine, milk and tears, as expected with IgA being the predominate immunoglobulin of mucosal surfaces. IgG was also abundant on mucosal surfaces. IgM was visualized in two bands suggesting that there may be subclasses in black flying foxes. As the first antibody to appear during an infection, it was hypothesize that more than one subclass may allow black flying foxes to mount a more robust first adaptive immune response to pathogens (124).

The basic structure of immunoglobulins is a pair of heavy chains and a pair of light chains comprised of a conserved constant region on the C-terminus and a variable region on the N-terminus. The variable region has a unique amino acid sequence that facilitates binding to a unique antigen that is generated through site-specific recombination of germline gene segments—V, D, and J in the heavy chain (H) and V and J in the light chain (L). Germline diversity occurs in naïve B-cells, prior to antigen stimulation. The varying numbers of germline gene segments (V, D and J) are rearranged to produce diversity amongst primary antibodies (125, 126). For example, humans have 40 functional VH segments,

24 DH segments, and 6 JH segments that can be arranged to produce 5,760 different H chains. Somatic hypermutation (SHM) occurs after exposure to antigens. It is the product of single base pair mutations that occur in gene segments increase avidity and affinity of immunoglobulins to antigens (125-127).

The VH segments are spread amongst three clans, identified as I, II or III. Most mammals only have VH segments representative of one clan, i.e. pigs having VHIII genes only and sheep have VHII representatives. Rodents and primates are the exception with segments from all three clans. A transcriptomic investigation of the black flying foxes found 23 unique VH sequences spread amongst the three clans. The Malayan flying fox also has VH sequences belonging to all three clans (125). Little brown bats have more 236 different V_H3 family gene sequences, a minimum of 24 DH segments, and 13 JH segments, yielding more than 70,000 VDJ transcript possibilities (123, 128). A large number of expressed VH repertoires may allow the bat to immunologically defend itself from a greater number of antigens compared to those animals with fewer, and may possibly be the product of co-evolution of bats with numerous viruses (116, 123, 125, 128).

While there is evidence for robust germline diversity amongst different bat species, SHM seems to have less significance in the bat's defense against pathogens. Mutation frequency for the FR3 and CDR2 region of the VH3 genes from adult little brown bats was determined and compared to germfree fetal pigs. They both had similar, low rates of mutation frequency. This is to be expected in a germfree fetal pig that has not been exposed to pathogens and has not yet undergone SHM. For an adult bat, this is unique and may suggest that bats have limited SHM and therefore reduced affinity maturation (128). The inconsistent neutralizing antibody titers elicited by bats in infection studies corroborates these data (30, 49, 50, 129). It's possible that the great germline diversity in bats negates the necessity of SHM but may also impact viral-host ecology, decreasing avidity of antigen specific antibodies, and altering immunological memory.

1.3.2.4: Relationship Between Flight and Immunity

It has been proposed that there may be a relationship between the capacity of bat to maintain viruses with minimal disease and flight, being that bats are the only mammals capable of sustained flight. The two dominate hypothesis are (1) flight produces high amounts of reactive oxidative species (ROS) that may damage DNA so it was necessary to have a physiological system in place that mitigated or repaired damage created by ROS and (2) that flight emulates fever, and this fever is a means by which to control viral infection.

To test whether bats had exceptional means to mitigate the effects or ROS or repair DNA damaged by ROS, high-throughput sequencing was done on samples from a representative of the suborder Yinpterochiroptera, the black flying fox (*Pteropus alecto*) and a representative of the suborder Yangochiroptera, David's Myotis (*Myotis davidii*). Flight has a high metabolic expense, which may result in the accumulation of ROS. It was found that *ATM* and *c-REL* in both species, *TP53* and *BRCA2* in David's Myotis, and *LIG4* in the black flying fox were positively selected for. The proteins of these genes are proponents of DNA repair or apoptosis. *C-REL* belongs to the nuclear factor κB family, an important transcription factor in innate immunity. In both bats, the gene locus with the PYHIN gene family is absent. PYHIN is associated with cell cycle regulation and at least one gene is present in all other explored groups of eutherian mammals. Given that genes important for DNA repair and maintenance of physiological homeostasis were positively selected for, it may be that the evolution of flight facilitated viral maintenance (6).

Due to the seeming absence of the PHYIN gene in black flying foxes, additional searches were conducted in other species of bats. Whole genome sequences of ten different bat species were done: the Malayan flying fox, the black flying fox, the straw-colored fruit bat, greater horseshoe bat (*Rhinolopus ferrumequinum*), greater false vampire bat (*Megaderma lyra*), the little brown bat, David's *Myotis*, Brandt's bat (*Myotis brandtii*), the big brown bat, and Parnell's mustached bat (*Pteronotus parnellii*).

The PYHIN (PYRIN and HIN domains) gene family has immune sensors for intracellular DNA that can subsequently activate inflammasomes or interferon pathways. It is the only DNA sensor that triggers inflammasomes. All bats lacked the PHYIN gene family with the exception of Parnell's mustached bat, which had a partial sequence. Within this sequence there was a frame shift mutation with multiple premature stop codons to indicate that the gene was no longer functional. Evidence for an ancestral PHYIN gene family in one bat may suggest that bat families independently lost the gene. It is hypothesized that the loss of the gene family may have been the byproduct of flight as a means to decrease inflammation and mitigate interferon response to native DNA that had been damaged due to the ROS formed during sustained flight (130). Whether or not this hypothesis holds, the lack of the PHYIN gene family does have interesting implications for the host relationship with DNA viruses. However, all the high profile human pathogens that bats harbor are RNA viruses so it does not explain how bats are able to maintain the viruses of human interest.

The second hypothesis surrounding flight and immune function was that flight increases metabolic rate and body temperatures. These increases might mimic fever and cause natural, regular fluctuations in bat physiology, thereby controlling viral infection and explaining why bats maintain a vast number of viruses without apparent disease (131). Bat body temperatures may fluctuate in response to flight (131), but bats are heterotherms—regardless of whether they are temperate, sub-tropical, or tropical species (132). Body temperature in bats fluctuates due to ambient temperatures, seasonality, and the weather (132). Non-hibernating pteropid bat temperatures may range from 33°C to 37°C throughout the day. Hibernating bat species' body temperature may drop to 5°C during hibernation (133). These numbers, however, are just examples. Temperatures are so variable across studies that it is difficult to identify normal bat temperature (132, 134, 135). Furthermore, studies determined that ambient temperature may be the ultimate determinant in body temperature during flight, rather than the metabolic expense of flying itself (134, 135). Additionally, the dominant mechanism for bats to disperse of heat is via cutaneous heat loss, and bats can actively thermo-regulate through wing tension. When a bat is in

flight, and the wing membrane is tight, they have maximum skin exposure to optimize heat dispersal (135). The act of flight is the major means by which bats cool down. Thus, to have flight mimic fever is contradictory to bat physiology.

Fever, in response to infection, is triggered by interleukin (IL)-1, IL-6, and tumour necrosis factor (TNF) that act on the hypothalamus to induce the production of cyclooxygenase-2 (COX-2). Prostaglandin is in turn produced which increases the body's temperature (127). Given that fever in response to infection is a complicated immunopathway, the idea that flight simulates fever has received some criticism (116). As heterotherms, fluctuations in body temperature are normal (132), and this is not synonymous with fever.

Fluctuations in body temperature have been demonstrated to effect viral-host ecology in bats, but not in a response to elevated temperature, but rather decreased body temperature. A coronavirus was isolated from North American big brown bats and little brown bats, and infection was persistent for four months while the bats were in hibernation (136). A different study explored this same coronaviruses in a similar population of non-hibernating bats and failed to find viral persistence suggesting viral maintenance varies between seasons (137). Silver-haired bats (*Lasionycteris noctivagans*) experimentally inoculated with different RABV strains showed prolonged viral incubation periods and viral latency during hibernation compared to non-hibernating bats (138). Hibernation is the result of a decrease in metabolic function, not merely body temperature and so without further exploration it cannot be concluded that the drop in body temperature alone facilitates microbe persistence (139).

1.3.2.5: Viral Mechanisms to Dampen the Immune Response

The evolution of virus-host relationships demands adaptation and the out-maneuvering of the opponent's defenses for survival. Bats and their viruses may have co-evolved for immense measures of

times, and being so both the immune system of the bat and the viral adaptions to the host must be highly adept at compensating for the other's evolutionary defenses (96).

Numerous zoonotic viruses of bats have accessory proteins that antagonize innate immunity in humans. ORF 3b, ORF 6 and N protein of SARS-CoV; the V, W and C proteins of Nipah virus; VP35 and VP24 of Ebola virus; and VP40 of Marburg virus interfere with IFN production. Furthermore, downstream effectors if IFN are inhibited. ORF 6 of SARS-CoV; V protein of Nipah; VP24 of Ebola and VP40 of Marburg viruses antagonize STAT1 in the Jak/STAT pathway (140-142).

An investigation of cell response to henipaviruses using primary *P. alecto* foetus cells (PaFe), primary *P. alecto* kidney cells (PaKi), immortalized *P. alecto* lung cells (PaLuT02), and immortalized *P. alecto* fetus cells (PaFeT) was conducted. Transfection with polyI:C was used as a positive control. Results demonstrated that cells inoculated with virus had a decreased expression of type I and type III IFNS that were comparable to mock infected cells, as determined by PCR to measure mRNA, and ISG production was also suppressed, specifically ISG54 and ISG56. *In vitro* research in human cell lines also demonstrated IFN antagonism in response to virus; however, the viral protein that antagonizes the response between bats and humans seems to be different for Hendra virus. In human cell lines it is the protein products from the viral P-gene, but not so in bat cell lines, but the mechanism in bat cells has yet to be elucidated (143).

Myxovirus resistance (*Mx*) genes are ISGs that respond to viral infection by disrupting viral structures. *Mx1* genes were cloned from six different immortalized bat cell lines belonging to Seba's short-tailed bat, Daubenton's bat (*Myotis daubentonii*), common pipistrelle bat (*Pipistrellus pipistrellus*), straw colored fruit bat, hammer-headed bat, and Egyptian fruit bat. Mx1 expression had a suppressive effect on Ebola virus polymerase function, especially Mx1 of straw colored fruit bat—the purported

reservoir for Ebola virus. In addition to Ebola virus, bat Mx1 antagonized viral polymerase activity for *Rhabdoviridae* and *Bunyaviridae (144)*.

In R06Ej cells (immortalized fetal Egyptian fruit bat cells) and RoNi/7 cells (immortalized kidney Egyptian fruit bat cells) infected with EBOV and MARV, generates a more robust innate immune response for MARV infected cells than EBOV infected cells. At 24-hours post infection with EBOV, IFN and ISG expression was relatively unchanged in R066EJ cells and antagonized in RoNi/7 cells. MARV infection in these same cell lines resulted in an increase expression if IFN-γ and IFN-λ as well as ISG54 and ISG56, but did demonstrate a suppression of type I IFNs. Viral accessory proteins VP35 and VP24 are critical for suppressing the innate immune response (145).

This highlights that relationships between viruses and different hosts varies on a molecular level, which would be expected for a reservoir host that does not have disease in response to infection and may have co-evolved with the viruses for great lengths of time, compared to an incidental host that hat severe disease.

1.3.3: Susceptibility to Disease

While there is a massive amount of data to support that bats are capable of maintaining numerous viruses without apparent disease, it is important to state that bats are not immune to disease from infectious agents. They are a mammalian species with the same over-arching mechanisms of immunity.

Since 2006 white-nose syndrome has claimed an unprecedented number of bats in North America, upwards of millions, resulting in the fastest decline of an animal species in history. It is a disease caused by *Pseudogymnoascus destructans*, a psychrophilic fungus that infects hibernating bats. The fungus affects the skin of the nose and ears, and wing and tail membranes, which results in water and electrolyte imbalances and may be the cause of premature waking from hibernation in the midst of winter

(146). The immune function is down regulated in a normal, healthy, hibernating bat and while wing lesions have few infiltrations of immune cells, infected bats demonstrate leukocytosis on complete blood counts. The fungus most likely came to North America via human travel from Europe (147, 148).

Due to the rapid decomposition of carcasses, it can be difficult to determine the cause for mortality events amongst bat species, and many infectious disease instigated die-offs may go undiagnosed (147, 149, 150). Outside of white-nose syndrome, a recent review recorded 25 multiple mortality events, worldwide, in bats caused by infectious diseases (147). *Salmonella* Enteritidis and *Salmonella* Typhimurium have been isolated from Vespertilioinidae bats. Histopathology revealed the bats had interstitial pneumonia and purulent meningitis, amongst other inflammatory lesions (149). Additionally in Vespertilioinidae bats, *Clostridium perfringens* and *C. sordellii* have been documented to cause hemorrhagic diarrhea in Europe, and *Escherichia coli* can cause urinary tract infections (149). *Borellia* can cause disease and pathology (151). A pipistrellus bat was found moribund in England and died a few days later from hepatitis and septicemia caused by a novel species of *Borellia (152) Pasteurella multocida* serotype 1 is the top differential for a mortality event that occurred in big brown bats in Wisconsin in 2008. Five carcasses were used as a representative population with multifocal pneumonia and diffuse serosal congestion or hemorrhage of the intestines appreciated on gross necropsy and coccobacilli visualized in vascular lumens of the lungs, liver and spleen. Cause of death was described as septicemia that produced an interstitial pneumonia with *P. multocida* isolated from lung, liver, spleen, heart and mammary gland tissues (153).

Viruses too, can cause pathology and disease in bats (151). A novel adenovirus, bat adenovirus 2 strain *P. pipistrellus* virus 1 (AdV-2), was identified by virus isolation on Vero E6 cells and electron microscopy in intestine, liver, kidney, and lung of *Pipistrellus pipistrellus* bats that were found moribund and later died. Tissues decomposed too rapidly to accurately assess histopathology but given that no other viral or bacterial agents were identified it was concluded that AdV-2 may have been the cause of the

bat's moribund state and death (150). Tacaribe virus (TCRV) is an arenavirus first isolated from Artibeus bats in the 1950s. During a rabies virus investigation, TCRV was isolated from bats with neurological disease when no rabies virus was found (154). Nearly fifty years later, an experimental inoculation of Jamaican fruit bats with TCRV resulted in neurological and respiratory disease with wide tissue dissemination of the virus, and lesions characterized as mild to moderate neutrophilic interstitial pneumonia, lymphocytic leptominingitis, gliosis, and neutrophilic encephalitis (129). Not to mention the well-established lyssavirus pathology with which mortality ranges from 6% to 40% in experimentally inoculated bats (30). Infected bats have clinical disease and on histopathology meningoenchalomyelitis and nonsuppurative ganglioneuritis are seen (31).

1.3.4: Ecological Perspectives on Bats as Reservoir Hosts

A trait-based analysis that used generalized least squares demonstrated that sympatry in bats is a trait strongly associate with zoonotic infection (38, 39). Sympatry in rodents is also a significant correlative trait to the number of zoonotic viruses; however rodent sympatry does not occur to the same degree as bat sympatry. Species mingling in rodents occurs when territories overlap. Mixed species in bats occurs because it is a behavioral norm for the gregarious order to roost in high densities with numerous species. In fact the effect of sympatry as it correlates to disease emergence is nearly four times greater in bats compared to rodents (38). Given that there has been a natural case of species hybridization in bats (12), and limited molecular phylogeny conducted on bats to concrete taxonomic relationships, it may be that bats of different species interbreed on a regular basis and this produces a greater rate of bat genomic variation. This could be a factor in co-evolution between the hosts and the viruses.

Numerous bat species, of both temperate and tropical varieties, migrate (155, 156). North American species of bats will migrate south for the winter or migrate dependent on seasonal food availability. Tropical bat species have been documented to migrate to breeding grounds (155). African straw colored fruit bats, the purported reservoir for Ebola virus, travel more than 2,000 km in three

months, and up to 500 km within a few days' time. Migration is motivated by spatial and temporal availability of fruit, traveling to those areas where fruit is most abundant (156). The fact that bats are capable of flight, allowing for them to cover large distances, may facilitate viral spread to both naïve bat populations and/or to areas where human-bat interactions are heightened (97, 98, 156).

1.4: Anthropogenic Change Facilitates Viral Emergence

There is an unprecedented amount of research directed towards bats as "special" and the mechanism by which they are exceptional reservoir hosts. The difficulty in the word "special" is that it infers a human-centric perspective on disease emergence. It asks what is different about bats that allow them to have such a substantial impact on human health, but does not consider the reverse. What is "special" about humans that facilitates disease emergence from bats? When the focus shifts from bats as "special" to humans as "special" it becomes easy to ascertain alterations in human activity that have occurred since the documented emergence of Marburg virus in 1967, and subsequent emergence of the numerous bat-associated human pathogens since.

With more than seven billion people on Earth and the exponential growth in human population, the line that separates human territory from natural habitats becomes ever-increasingly blurred and interactions between humans and wildlife surge. Resource consumption intensifies in order to provide for the number of people on Earth. Tropical deforestation occurs at a global rate of 2-3% with land use driven by agriculture, lumber harvesting, road construction, urban expansion, mining and more (157). Between 1990 and 2015, 129 million hectares of forest have been lost. This is an area the size of South Africa (158). Loss of habitat is predominantly due to deforestation and may make previously secluded bat populations more accessible for hunters. Logging and slash and burn techniques directly decrease bat habitats but also, combine with human landscape alteration, alters environmental landmarks, such as waterways and tree lines, that bats may use to navigate from one area to the next (159).

Alterations of the natural landscape for human use will bring humans markedly close to wildlife species and with 75% of human emerging infectious diseases tied to wildlife (160), this may make people vulnerable to an even greater number of EIDs (161). Expansion in urbanization and increased contact between bats and humans is identified as a major risk for viral spillover (39, 162). The emergence of Hendra virus into human populations is linked to anthropogenic change. Bats took advantage of human food sources, including fruit trees planted in horse paddocks. Consequently bats migrated less and local populations increased four-fold between 1999 and 2014, increasing interactions between bats, horses, and humans that created an opportunity for Hendra virus emergence (162). Interesting that it is the lack of migration that resulted in disease emergence when it is the migratory capacity of bats that is implicated in disease spread. It may be that a deviation from normal behavior due to human interaction is the greater risk for viral exposure and spillover.

Carbon emissions have increased 90% since 1970. Seventy-eight percent of this is due to fossil fuel combustion from human use (163). Between 1940 and 2006, the global mean surface temperature increased 0.55°C (164, 165). 2016 was the third year in a row to set a new record for global average surface temperature, driven by a rise in carbon emissions (166). Rising temperatures result in rising sea levels, distorted seasonality, and altered landscapes (164, 165).

The combination of climate change and deforestation were driving factors in the emergence of Nipah virus. Epidemiological studies traced the emergence of Nipah virus to anthropogenic forest burn exacerbated by El Nino event in 1997—1998. Anthropogenic burning is one of the greatest threats to rainforest destruction. The intention is to clear areas of land for industrial crops or burn agricultural waste. In 1997 rainforest in Kalimantan and Sumatra were burned (167). At this same time was a record holding El Nino Southern Oscillation event. Every few years, over the tropical eastern Pacific Ocean, there are fluctuations between warming and cooling phases in combination with high and low air surface pressure. This pattern is referred to as an El Nino Southern Oscillation and the subsequent effects can be

variable from drought to flooding in the tropics and subtropics (168). The 1997—1998 El Nino event resulted in drought in Southeast Asia. The anthropogenic fires were exacerbated by the drought, resulted in five million hectares of land burned, and a dense haze that was most severe in south Malaysia in the region of the first Nipah virus outbreak. While it is unclear the full extent that such air pollution has on the environment, oral local histories declare that there was severe crop failure due to the haze and so it is possible to assume that rainforest flowering and fruiting was decreased as well. Loss of habitat, and decreased flowering and fruiting altered natural fruit bat ecology, and may have forced bats to migrate to cultivated orchards. Prior to 1997 and 1998, Malayan flying foxes were not observed in the orchards that surround pig farms by local farmers, after 1998 they were, and fruit partially eaten by bats had been found in pigsties—offering a route of exposure of pigs to bats and Nipah virus and ultimately spillover into humans (167).

Analysis predicts that 15% to 37% of plant and animal species will be extinct by 2050 due to climate change (169). We are currently in Earth's sixth mass extinction. Species are being lost at an unprecedented rate. Over the last century, conservatively, 200 species of vertebrates have gone extinct. Measured against the background extinction rate that has occurred over 2 million years, these 200 species should have taken 10,00 years to disappear. The rate of loss of biodiversity is attributed to deforestation, consumption of natural resources, toxification, disease, and climate change, but ultimately the product of human over-population and over-consumption (170). While climate change is considered a factor in species loss, a trait-based analysis demonstrated that prior mass extinctions had a uniform distribution of loss regardless of body size as it pertains to oceanic species. Larger-bodied species were lost at the same rate as small-bodied species. Losses in the modern oceans have all had large body masses, indicating that we have not yet seen loss due to climate change. Climate change results in the acidification of oceans and subsequent loss in benthic marine micro fauna, which leads to nutrient pollution, and a lack of oxygen in bottom waters producing dead zones. The impact of these dead zones on species decline does not yet

compare to species decline as a product of human encroachment and over-fishing. The negative impact of climate change has not yet fully started (171).

Illegal wildlife trafficking is ranked among the top ten most profitable black-market trades and is estimated to generate between $7 and $10 billion annually. It is actualized in the form of poaching and trapping to sell bush meat, prized wildlife parts, and live animals for the exotic pet trade (172). It is estimated that tropical wildlife species are being hunted for bush meat six times the sustainable rate (173). A review of the multiple mortality events (MMEs) in bat that have occurred globally since 1790 determined that the number one cause of bat losses is anthropogenic in origin, and account for 54% of all MMEs, in particular wind turbines. While wind turbines remain the number one cause of MMEs, documented intentional killings of bats by humans account for 205 events out of 1180 (147).

Intentional killing of bats predominates in the Eastern Hemisphere—where all of the non-rabies bat-related EIDs originate—compared to the Western Hemisphere. People kill bats for food in Asia and Africa. In Africa, straw colored fruit bats are the species of choice, the proposed reservoir for Ebola virus (147), and encroachment on bats may have instigated the emergence of Ebola and Marburg virus (174). In Asia bats are frequently taken to animal markets. The spillover event of SARS-CoV from the incidental host, masked palm civets (*Paguma larvata*) to humans occurred in Xinuan Live Animal Market in Guangdong Province, China (175, 176). In Borneo in 2003, 4500 large flying foxes (*P. vampyrus natunaei*) were killed for food in one day (147). On Niue Island in the South Pacific, 1555 pteropus bats were shot between 1998 and 1999; an estimated 38% to 76% of the population (173). Anecdotally, in North Sulawesi culture in Indonesia, flying foxes have been consumed for decades. In the 1970s the economic benefit of selling bats in markets was appreciated, and instigated an increase in bat hunting. As local bat populations dwindle, the rate of importation of flying foxes from Borneo increased so that locals never appreciate a market deficit and bat population decline. Approximately 45 to 75 flying foxes are sold daily for food on Tuesday through Friday, with more on Saturdays and Mondays when 150 to 445

bats are sold. The bats that are most frequently eaten in Indonesia are listed as CITES Appendix I or II animals, but there is no local enforcement to prevent hunting (177). Pteropid bat hunting has doubled in Malaysia since 1996 (173).

In Australia pteropid bats are shot, trapped, exterminated with explosives, burned, and electrocuted with electric grids. Millions of bats have been killed in the name of crop protection (147). In Europe and North America, bats are terminated by similar means as in Australia but also via fumigation, although documentation suggests to a lesser extent than in Australia. In South America, where rabies transmission from vampire bats has become a public health threat, bat killing occurs indiscriminately of species with fumigation, electrocution, intentional disease introduction, shooting, and trapping (147). Bats are long-lived animals with a low reproductive rate and replacing their losses will take a long time (178). Of the more than 1100 bat species, the International Union for the Conservation of Nature (IUCN) lists 18 as Critically Endangered, 40 as Endangered, and 83 as Vulnerable (179-182).

1.5: Conclusion

There is no doubt that bats harbor more zoonotic pathogens than any other species. Elucidating the mechanism by which this occurs may reveal a means by which intervention can be implemented and decrease risk of spillover. The principle ecological trait that has been revealed to correlate to the ability to harbor zoonotic pathogens is sympatry—inter-mingling of species, yet a real-world demonstration of how this has impacted disease emergence has yet to be seen. The immunological profile of bats suggests they constitutively express type I IFN. This imparts that a major immune defense that is typically activated in the face of infection is already "on" in bats and may mitigate viral infection and propagation. Furthermore, bats may not undergo somatic hypermutation, which would be a weakness in their learned immunity and decrease affinity maturation. This may be a mechanism by which bats can circulate and re-circulate virus. It's important to note that what is determined in one bat species may not be transferable to

another bat species. More research needs to be conducted to determine how evolutionarily conserved physiology is across bat species.

Ultimately the reasons behind disease emergence are a tangled web of viral ecology, host ecology, natural ecology, and human encroachment where one size may not fit all. Delineating a clear explanation will take much concerted effort. By exploring novel viruses in Jamaican fruit bats and studying the resultant pathology we hope to add to the repertoire of data to understand the role bats play in disease emergence.

1.6: Rationale for the Current Study

The overall aim of the study described in the next four chapters was to better understand disease processes of novel viruses in bats. A captive Jamaican fruit bat colony (*Artibeus jamaicensis*) is maintained at Colorado State University, and the following two chapters used these bats to conduct experimental inoculations of bat influenza virus and Zika virus to elucidate pathophysiology. Expanding upon experimental inoculations, we also conducted field research and serological investigations for evidence of Tacaribe virus in Trinidadian fruit bats. The final chapter of this work details *de novo* sequencing of the Jamaican fruit bat genome to lay the groundwork for future projects that require bat specific reagents.

H17N10 and H18N11 influenza A viruses were discovered in New World bats in 2010 and 2011 (181, 182). These viruses are highly divergent from other influenza A viruses and incite intrigue as to the role bats play in influenza virus ecology (179-182). This chapter describes the use of rescued bat influenza viruses to conduct the first *in vivo* bat investigations since discovery of the viruses. Route of transmission and susceptibility of naïve bats were included in overall study design. This work is described in chapter two.

Zika virus (ZIKV) was first introduced into the New World in 2014 and quickly spread throughout South America, Central America and the Caribbean (183, 184). Limited research was conducted in the 1950s and 1960s that explored susceptibility of bats to Zika virus, and found bats could be infected (179-182). Given that Jamaican fruit bats are endemic to a spatial geography that overlaps with current presence of Zika virus in the Western Hemisphere, and that both wild New World bats and Old World bats have been found with evidence for dengue virus and chikungunya virus infection (viruses related to ZIKV) (13, 185), we sought to investigate the susceptibility of Jamaican fruit bat to ZIKV to ascertain if they might be a reservoir host. Dengue virus, chikungunya virus and ZIKV are all vectored by *Aedes* mosquitos, and evidence of dengue and chikungunya virus infection in bats implies that the vector for ZIKV will feed on bat species (179-182, 186). Additionally, experiments were conducted with postulation that these bats may serve as a unique animal-model for ZIKV infection. This work is described in chapter three.

The experiments described in chapter two and chapter three both investigate the adaptive immune response, potential morbidity, gross pathology and histopathology, viral tropism, and time course of disease to their perspective viruses. Taken together these experiments further the understanding of pathophysiology in bats and expand knowledge on virus-host ecology. These two virus types in bats may be usable as models to better understand the role of bats as reservoirs, given the viruses cause minimal pathology.

Chapter four details the serological investigation of Tacaribe virus (TCRV) exposure in wild-caught fruit bats. TCRV is a New World mammarenavirus that was first isolated from moribund bats and a single mosquito pool in the 1950s (154). Serological studies in the 1960s surveyed more than 2,000 animal species and found bats to be the only animals with antibody titers to TCRV (187). TCRV was not isolated again until just recently where it was discovered in questing amblyomma ticks in Florida (188). Because TCRV was first discovered in bats, bats were the purported reservoir species—remarkable given

that rodents are the reservoir for multiple arenaviruses, not bats. The research described in this chapter seeks to identify if TCRV is still circulating in bats in Trinidad.

CHAPTER 2: INFECTION AND PATHOLOGY OF BAT INFLUENZA VIRUSES IN JAMAICAN FRUIT BATS (*ARTIBEUS JAMAICENSIS*)

2.1: Introduction

In 2009 and 2010 two novel influenza viruses were discovered in two different bat species in South America. Later classified H17N10 and H18N11 for the newly discovered hemagglutinin and neuraminidase glycoproteins, these viruses align most closely with group one influenza A viruses (IAV), yet are highly divergent (181, 182). Because these viruses were detected in animals that garner much scientific attention, and because the viruses deviate from canonical understanding of influenza viruses, many questions are raised; are bats a natural reservoir of influenza viruses, is zoonosis a risk, what information can be gleamed in regards to influenza virus evolution (179, 180)?

The H17N10 IAV was discovered in rectal swabs collected from three little yellow-shouldered bats (*Sturnira lilium*) out of 316 rectal swabs collected from 21 different bat species in Guatemala. Samples were collected in 2009 and 2010 and positive samples detected by qRT-PCR using pan-influenza primers (181). The H18N11 IAV was discovered in samples collected from a flat-faced fruit bat (*Artibeus planirostris*) in 2010 in Peru. Of the 110 animals screened from 18 different species, one flat-faced fruit bat rectal swab, and correlating intestinal tract were positive via qRT-PCR using pan-influenza primers. Fifty-five of the 110 animals tested were seropositive for H18N11 (182). These are the only publications that describe the identification of bat influenza viruses in wild populations of bats. The viruses themselves were not isolated (181, 182). Next generation sequencing with *de novo* assembly and RT-PCR amplicon-based Sanger sequencing identified the viral genome sequences of both viruses (181, 182).

The hemagglutinin (HA) glycoprotein of IAV facilitates virus attachment to the sialic acid receptor on host cells, as well as fusion of viral and cell membranes for viral entry. Neuraminidase (NA)

glycoprotein functions in viral release by cleaving sialic acid from viral glycoproteins (103). Structural analysis revealed that H17N10 and H18N11 HA and NA proteins are similar to their related counterparts; however, there are critical differences (182, 189-192). The receptor-binding cavity of H17 and H18 is much smaller than other HAs, which may inhibit binding with sialic acid. Additionally, in other IAVs, uncharged residues permit binding to the negatively charged sialic acid, but in bat influenza HAs these same residues are negatively charged and may repel sialic acid (182, 190, 192). If bat influenza HAs do not bind sialic acid then bat influenza NAs may not need to function as a neuraminidase (193). While bat influenza NAs share the same homotetrameric structure as the established IAV NA, bat influenza NAs have a wider 150-cavity—an additional cavity adjacent to the active site of almost all group one NAs. Furthermore, the established conserved active site and framework amino acids vary in bat influenza NAs (182, 189, 191, 194).

Structural suppositions were corroborated with experiments that used both pseudotyped and rescued bat influenza virus. Protease-treated rhabdoviruses, with native glycoproteins deleted and expressing bat influenza glycoproteins, yield limited cell tropism. Human, monkey, bat, canine, porcine and avian cell lines failed to support viral infection and propagation. There are a few cell lines that permit viral propagation including bat cell lines, MDCK-II, and RIE 1495 cells (195-197). Pseudotyped virus research has allowed for the acquisition of the following information: HA cleavage is necessary for cellular infectivity, HA mediates attachment and entry, a low pH may be required for membrane fusion, and bat influenza HAs use a receptor other than sialic acid (195, 196). Reverse genetics to rescue all eight gene segments of influenza viruses was first established in 1999 and has since become an established practice in influenza virus research (198-200). The rescue of H17N10 virions, propagated through reverse genetics, supported research using pseudotyped viruses, and indicated an inability to infect canine, mink, porcine, primate, human and bat cell lines (201). Combined with pseudotyped virus cell culture results, this highlights the species specificity of bat influenza viral glycoproteins.

The majority of the research conducted to date to characterize bat influenza viruses has occurred *in vitro*. No research has been conducted *in vivo* in any bat species. With the successful generation of H17N10 and H18N11 viruses through reverse genetics, generously donated by Dr. Wenjun Ma at Kansas State University, we were able to investigate susceptibility of Jamaican fruit bats (*Artibeus jamaicensis)* to both rescued H17N10 and H18N11 viruses. The objective of the studies presented here were to determine susceptibility, disease characterization, pathophysiology, organ burden and antibody response to the viruses. Given that bat cell lines from numerous bat families supported pseudotyped viral infection (196), we hypothesized that Jamaican fruit bats would be susceptible to infection. Moreover, we hypothesized viral tropism would target tissues of the gastrointestinal tract as bat influenza viruses were first identified in rectal swabs of bats (181, 182).

2.2: Methods and Materials

2.2.1: Bats

All bat procedures were approved by the Colorado State University (CSU) Institutional Animal Care and Use Committees and were in compliance with U.S. Animal Welfare Act. CSU maintains a captive colony of Jamaican fruit bats (*Artibeus jamaicensis*). The colony was first established at CSU in 2016 as a subset of bats from the University of Northern Colorado. The colony been closed since 2006. Bats are kept in a free flight room measuring 19'w x 10'l x 9'h. Roosting baskets are hung from the ceiling throughout the room and drapes of different cloth material are positioned for hanging and roosting. Ambient temperature is maintained between 20°C and 25°C, with humidity between 50% and 70%, and a 12 hour light/12 hour dark light cycle via a computer-controlled system. Bats are fed from multiple feeding trays once a day a combination of fruits, Tekald primate diet (Envigo, Huntington, UK), molasses, nonfat dry milk and cherry gelatin. Water is provided in a water tray. In addition, fruit is hung around the room to stimulate foraging behavior and serve as enrichment.

For infection experiments, bats were trapped using a butterfly net and placed in an 20" d x 12" w x 18" h cage for 24 hours prior to inoculations to allow for acclimation. Hanging clothes were provided for roosting and coverage. Food and water were placed in open trays in the bottom of the cage and changed daily. Tray liners were changed every two days, and cages and hanging clothes were changed every two weeks. Due to the social nature of these bats, a minimum of two bats were kept in cages at all times to mitigate potential stress.

2.2.2: Virus

Bat influenza viruses have not been isolated from the wild (181, 182). Reverse genetics was used to rescue both bat influenza viruses (H17N10 and H18N11), and were generously provided by Dr. Wen Jun Ma at the Department of Diagnostic Medicine/Pathology, College of Veterinary Medicine, Kansas State University (KSU), Manhattan, Kansas, USA (201).

2.2.3: Experimental Infection—H17N10

A pilot study using male bats was conducted to determine susceptibility of Jamaican fruit bats to H17N10 IAV. Male bats were used in both pilot studies because females are prioritized for breeding and colony expansion. Bats were anesthetized with 1% to 3% isoflurane to effect with an oxygen flow rate of 1.5 L/min, administered with a gas mask. Animals were placed on a heating pad to maintain body temperature and respirations monitored continuously. Twenty-five microliters of media containing of H17N10 was inoculated intranasally with a micropipette—12.5ul into each nostril. Due to the limited tropism in cell culture, a cell culture system had not yet been optimized to determine virus titers. Bats were held upright for one minute to prevent inoculum from draining out of nostrils. When procedures were finished bats were removed from isoflurane and placed back in the cage in ventral recumbency. Respirations were monitored until animal was fully awake and ambulated normally. Animals were euthanized at 28 days post-inoculation (DPI). All procedures were performed in a biosafety cabinet at biosecurity level two facility.

2.2.4: Experimental Infection—H18N11

Two sets of experiments were performed; a pilot study and a time course study. For the pilot study, three male bats were anesthetized and procedures carried out with the same methods as for the H17N10 pilot experiment. Similar to H17N10, an initial viral titer for H18N11 had not yet been established for the pilot study. One of the bats died during recovery from anesthesia and gross necropsy performed. The cause of death was not determined but likely due to complications from anesthesia. The remaining two animals were euthanized at 28 DPI. All procedures were performed at a biosafety level two. Bats from this study are identified as AJ-1p and AJ-2p.

For the time course study 10 bats (5 male and 5 female) were anesthetized under the same protocol as the pilot studies. Animals were then placed in ventral recumbency and the dorsum of each animal was sprayed with 70% ethanol. IPTT300 transponders (BioMedic Data Systems, Inc., Seaford, DE) were inserted subcutaneously (sc) at the level of the caudal edge of the scapula following delivery of 0.15 mls of 1% lidocaine. Animal IDs were as follows: AJ-h1, AJ-h2, AJ-h3, AJ-h4, AJ-h5, AJ-h6, AJ-h7, AJ-h8, AJ-h9, and AJ-h10. Twenty-five microliters of H18N11 containing $7.5x10^5$ 50% tissue culture infective dose ($TCID_{50}$) was inoculated intranasally with a micropipette under isoflurane anesthesia as described above. Four bats (2 males, 2 females) were placed in one cage and six bats (3 males, 3 females) were placed in a second.

Two days post inoculation of AJ-h1 thru AJ-h10; two naïve bats (one male, one female) were anesthetized under the same protocol and IPTT 300 transponders inserted. These bats, identified as AJ-t11 and AJ-t12, were not inoculated with H18N11. AJ-t11 and AJ-t12 were used to study the natural transmission cycle of H18N11, and were placed in the cage of four inoculated bats to be exposed to experimentally inoculated animals. The transmission bats remained in the cage with inoculated bats for the duration of the study.

AJ-h1 (male) and AJ-h2 (female) were euthanized at 3 DPI. AJ-h3 (female) and AJ-h4 (male) were euthanized at 6 DPI. AJ-h5 (male) and AJ-h6 (female) were euthanized at 10 DPI. AJ-h7 (male) and AJ-h8 (female) were euthanized at 17 DPI. AJ-h9 (male) and AJ-h10 (female) were euthanized at 28 DPI along with AJ-t11 (male) and AJ-t12 (female)—the two transmission bats. The transmission bats were exposed for a total of 26 days before euthanasia. Experimental design, including time points for euthanasia, necropsies, and tissue collection, and animal identifications are diagramed in figure 2.1.

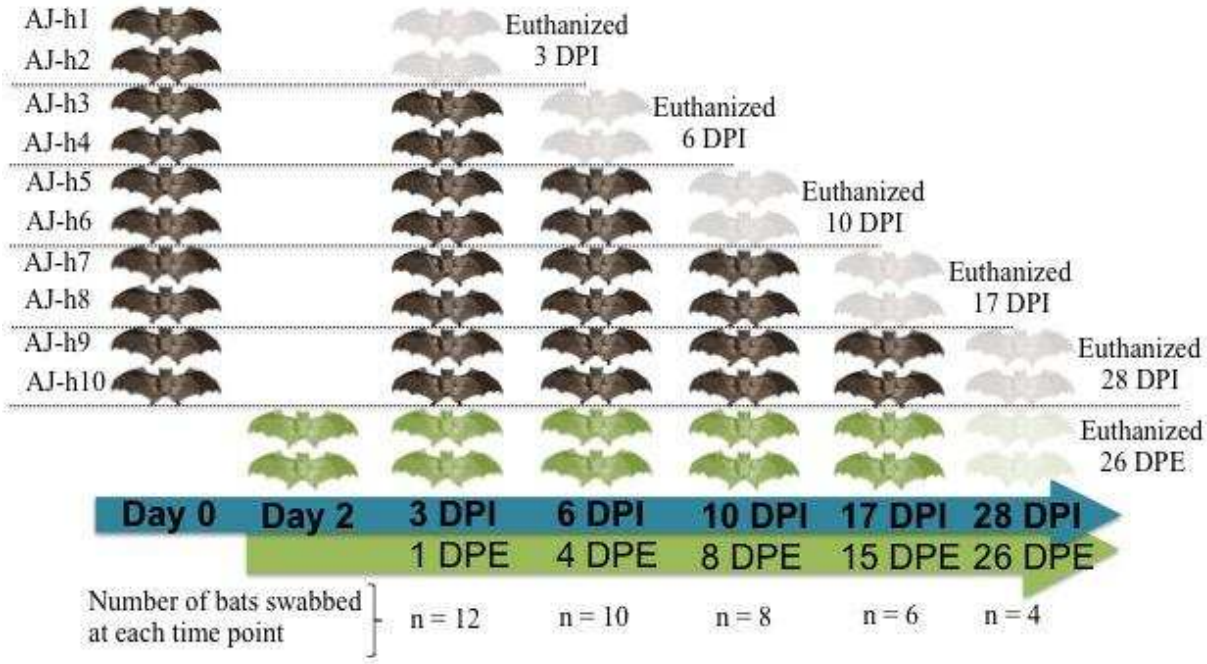

Figure 2.1: Diagram of experimental design. AJ-h1 thru AJ-h10 represent bats inoculated with H18N11 on day 0. Green hued bats represent AJ-t11 and AJ-t12, the transmission bats exposed to inoculated bats two days after inoculation. Translucent bats indicate animals that were euthanized with euthanasia time points immediately to the right. Blue arrow indicates time points swabs were collected for inoculated bats, green arrow indicates time points swabs were collected for transmission bats. The total number of swabs collected at each time point is listed under the arrows.

2.2.5: Monitoring and Swabs

Bats were monitored twice a day through the duration of the experiment for any deviations from normal. In particular food intake, presence of lethargy, inability to ambulate, respirations, nasal or oral discharge, and fecal consistency were assessed.

For both the H17N10 and H18N11 pilot studies oropharyngeal and rectal swabs were collected 2, 4, 7, 15, 21 and 28 DPI. For the H18N11 time course study, rectal swabs were collected from all existing animals at 2, 3, 6, 10, 17 and 28 DPI for inoculated bats. Because of the study design—transmission animals added two days after inoculation—the transmission bats had rectal swabs collected at 1, 4, 8, 15, and 26 days post exposure (DPE). With euthanizing two inoculated bats at each pre-determined time point, inoculated animals had rectal swabs taken as follows: all 10 bats at 3 DPI, 8 bats at 6 DPI. (AJ-h3, AJ-h4, AJ-h5, AJ-h6, AJ-h7, AJ-h8, AJ-h9, AJ-h10), 6 bats at 10 DPI (AJ-h5, AJ-h6, AJ-h7, AJ-h8, AJ-h9, AJ-h10), 4 bats at 17 DPI (AJ-h7, AJ-h8, AJ-h9, AJ-h10), and 2 bats at 28 DPI (AJ-h9, AJ-h10). Time points for swabs are diagramed in figure 2.1. For rectal swabs, a sterile polyester tipped applicator (Puritan, Guilford, ME) was inserted into the rectum and swirled gently. For oropharyngeal swabs, a sterile polyester tipped applicator (Puritan, Guilford, ME) was inserted into the oral cavity and swirled gently. Applicators were placed in 500 ul of sterile brain heart infusion broth (BHI) (Millipore Sigma, St. Louis, MO) prepared according to manufacturer's directions. Swabs were stored at -80°C until use.

2.2.6: Euthanasia and Necropsy

Bats were anesthetized and maintained with 3% isoflurane and an oxygen flow rate of 1.5 L/min. Depth of anesthesia was assessed by firmly pinching skin and toes to test for response. When deep anesthesia was achieved a thoracotomy was performed by puncturing through the skin, skeletal muscle, and diaphragm just caudal to the sternum with sterile standard scissors. A cut was made through the chest cavity to allow the heart to be visualized. Bats were maintained on isoflurane until breathing ceased for one minute.

Cardiac blood was collected with a 21 gauge sterile hypodermic needle. A total blood volume of between 1 and 1.5 ml was collected in a syringe and transferred to a serum separator tube (SST). SSTs were inverted approximately 10 times, allowed to sit at room temperature for one hour for clot formation and centrifuged at 1000 x g for 10 min at room temperature. Serum was removed from the clot, placed in a sterile microcentrifuge tube and stored at -20°C.

Necropsies immediately followed euthanasia. Sterile instruments were used to collect heart, lung, liver, spleen, gastrointestional tract, reproductive organs, kidneys, bladder, mandibular salivary glands, nasal turbinates, brain and tonsils from all animals. A 1:10 w/v portion of the tissue was immersed in 10% buffered-formalin. A second portion of tissues were placed in microcentrifuge tubes and stored at -80°C for PCR.

2.2.7: Negative Control Bats

One male and one female bat (identified as AJ-NCm and AJ-NCf) were trapped from the colony and kept in the same type of cage as used to house bats for experimental infections. Bats were housed in the same conditions as experimental animals and euthanized after six days of being kept in the cage. Necropsies were immediately performed and tissues and blood collected. Euthanasia, necropsy and blood collection procedures were the same as for inoculated and transmission animals.

2.2.8: RNA Extraction

RNA extractions were performed using TRIzol Reagent according to Ambion Life Technologies protocol for tissues and swabs. For swabs, applicators were vortexed in the BHI with which they were stored. Two-hundred microliters of BHI was used per one ml of TRIzol Reagent. For tissues, one ml of TRIzol Reagent was added to approximately 50 mg of tissue and a 5 mm stainless steel bead (Qiagen, Valencia, CA). Tissues were homogenized with a TissueLyser LT (Qiagen, Valencia, CA) at 50 Hz for 5 minutes. Samples were then incubated at room temperature for 5 minutes. Two-hundred microliters of

chloroform (Thermo Fisher Scientific, Waltham, MA) was added, samples vortexed, incubated for 3 minutes at room temperature, and centrifuged at 12,000 x g for 15 minutes at 4°C. The aqueous phase was removed, placed in a new microcentrifuge tube, and 4 ug of glycogen (Thermo Fisher Scientific, Waltham, MA) with 500 ul 100% molecular grade isopropanol added (Thermo Fisher Scientific, Waltham, MA). Samples were incubated at room temperature for 10 minutes and centrifuged at 12,000 x g for 10 minutes at 4°C. Supernatant was discarded and 75% molecular grade ethanol (Thermo Fisher Scientific, Waltham, MA) was added to the RNA pellet, samples vortexed briefly and centrifuged at 7500 x g for 5 minutes at 4°C. Supernatant was discarded and RNA pellets allowed to air dry for 10 minutes. RNA was re-suspended in RNase-free water and stored at -80°C.

2.2.9: qRT-PCR

Probe-based quantitative reverse transcription PCR (qRT-PCR) was performed with primers and probes that target the nucleocapsid protein (NP) gene for both H17N10 and H18N11. Primers were designed using the published viral sequences (National Center for Biotechnology Information) and Primer Express 3.0 (table 2.1). The probe targeted a sequence that had 100% nucleotide identity between H17N10 and H18N11 so that the same probe could be used in either reaction. Roche Real Time Ready RNA Virus Master Kit (Roche, Indianapolis, IN) was used on according to manufacturer's instructions. Two-hundred nanograms of sample RNA was used per reaction and reactions were done in technical duplicates. A light-cycler 96 (Roche Diagnostics Corporation, Indianapolis, IN) was used. Amplification was performed according to manufacturer's protocol for Roche Real Time Ready RNA Virus Master Kit (Roche Diagnostics Corporation, Indianapolis, IN) with PCR conditions as follows: 8 min at 50°C, 30 s at 95°C, and 45 cycles of 10 s at 95°C, 20 s at 60°C and 10 s at 72°C. For the time course H18N11 study, log dilution series was made of the stock virus, which had a known $TCID_{50}$, and linear regression applied to determine $TCID_{50}$ equivalents.

Table 2.1: Primer-probe sequences for H17N10 and H18N11.

Primer Name	Sequence
H17N10 forward primer	5'-GAGAATCACAGACATGAGGACTG-3'
H17N10 reverse primer	5'-CCCTCGTCATTCCCATCTAGTGGA-3'
H18N11 forward primer	5' -AAGAATCACTGACATGAGAACTG-3'
H18N11 reverse primer	5'-CCCTCGTCATTCCCATCCAAAGAA-3'
Shared probe sequence	5'-FAM/CAACTAACC/ZEN/CGATAGTGCCT/3IABkFQ-3'

2.2.10: Histology—Hematoxylin and Eosin Stain (H&E)

Tissues were fixed in 10%-buffered formalin, cut in, and submitted to Colorado State University Veterinary Diagnostic Laboratory (CSU VDL, Fort Collins, CO) for paraffin embedding, sectioning and staining with hematoxylin and eosin.

2.2.11: Serology—ELISA

ELISAs were performed on serum collected from the H18N11 time course bats to assess for antibody against H18N11 nucleoprotein (NP). H18N11 NP antigen was generously provided by Martin Schwemmle from the Institute of Virology, University Medical Center Freiburg, Germany. Ninety-six-well tissue culture plates were coated with NP antigen at a concentration of 0.5 ug/ml antigen diluted in PBS. Plates were kept at 4°C for 12 hours and then washed twice with PBS, and blocked with 0.5% bovine serum albumin (BSA) for 30 minutes at room temperature. Plates were then washed twice with PBS. Bat serum samples were diluted 1:100 in PBS and then two-fold serial dilution series was performed with the highest dilution 1:3200. Fifty microliters of each dilution of serum was added in duplicates to wells. Plates were incubated for one hour at room temperature and washed three times with 0.5% Tween 20-PBS. HRP-conjugated protein A/G (Thermo Fisher Scientific, Waltham, MA) was added at a concentration of 2 ug/ml and incubated for 30 min at room temperature. Plates were washed four times with 0.5% Tween 20-PBS and 150 ul of ABTS Peroxidase Substrate (2 component) (KPL, Gaithersburg, MD) was added according to manufacturer's instructions and incubated for 30 minutes at

room temperature. One-hundred and fifty microliters of ABTS Peroxidase Stop solution (KPL, Gaithersburg, MD) was added and plates were read on an EMax Plus Microplate Reader (Cambridge Scientific, Watertown, MA) at OD 405. Limit of detectable response was set at three standard deviation values above mean negative control serum.

2.3: Results

2.3.1: Experimental infections – H17N10

A pilot study was conducted for H17N10 to determine if Jamaican fruit bats were susceptible to the rescued virus. Three male bats were experimentally, intranasally inoculated and monitored twice daily for 28 days for signs of clinical disease. No overt clinical signs of disease were observed throughout the duration of the study.

2.3.2: Experimental infections – H18N11

Two different experiments were conducted for H18N11: a pilot study and a time course study. For the pilot study, two male bats (AJ-h1, AJ-h2) were inoculated and monitored twice daily for 28 days for clinical disease. Bats did not exhibit any signs of clinical disease throughout the duration of the study.

The time course study used 12 bats. Ten bats, five male and five female (AJ-h1, AJ-h2, AJ-h3, AJ-h4, AJ-h5, AJ-h6, AJ-h7, AJ-h8, AJ-h9, AJ-h10) were inoculated with H18N11, $7x10^5$ $TCID_{50}$. Two additional animals that were not inoculated, one male and one female (AJ-h11t and AJ-h12t) were exposed to inoculated bats.

AJ-h12t had mild ocular and nasal discharge, and mild epiphora 6 days post exposure (DPE). Both AJ-h12t and AJ-h11t had mild ocular and nasal discharge and mild epiphora 15 DPE. No abnormalities were observed in transmission bats at other times, nor in any of the inoculated bats

throughout the duration of the study. Food consumption were within normal limits. Production of urine and feces were within normal limits.

One male and one female were used as negative control bats. Bats were housed in the same size cage as infection/transmission bats and received the same husbandry as infected animals. Bats were visually assessed daily for any abnormalities, of which there were none, and were euthanized six days after being kept in the cage.

2.3.3: Euthanasia and Necropsy

The three bats in the H17N10 study and two bats in the H18N11 pilot studies were euthanized 28 DPI and necropsies immediately performed. No gross pathology was observed in any organs.

For the time course study with bats inoculated with H18N11, bats were euthanized and necropsies immediately performed. Gross pathology in the form of congestions was observed in the lungs at time points: 3, 6, 10 and 17 DPI. AJ-h9 had similar lung pathology at 28 DPI while AJ-h10 and the two transmission bat lung tissues were within normal limits. Serosanguinous pleural effusion was present in the thorax upon necropsy of AJ-h7 and AJ-h8, 17 DPI. Negative control animals had mildly congested lungs similar infected/transmission animals. Gross lung pathology is summarized in table 2.2.

Table 2.2: Gross lung pathology in time point study Jamaican fruit bats inoculated with H18N11 and negative control animals.

Days Post Infection/Exposure	Animal ID	Lung Congestion
3 DPI	AJ-h1	+
	AJ-h2	+
6 DPI	AJ-h3	+
	AJ-h4	+
10 DPI	AJ-h5	++
	AJ-h6	++
17 DPI	AJ-h7	-
	AJ-h8	++
28 DPI	AJ-h9	+
	AJ-h10	-
26 DPE	AJ-t11	-
	AJ-t12	-
Neg Cont (6 days post)	AJ-NCm	+
	AJ-NCf	+

- indicates no significant gross pathology. + indicates mild pathology. ++ indicates moderate pathology.

2.3.4: qRT-PCR

2.3.4.1: qRT-PCR H17N10

Quantitative probe based reverse transcription PCR (qRT-PCR) was performed on rectal and oropharyngeal swabs collected at 2, 4, 7, 15, 21 and 28 DPI. Viral RNA was not detected in any of the samples. Quantitative RT-PCR was performed on RNA extracted from lung, trachea, liver, small intestine, large intestine, spleen, and kidney collected 28 DPI. Viral RNA was not detected in any of the samples.

2.3.4.2: qRT-PCR H18N11

For AJ-1p and AJ-2p, the pilot study bats, quantitative probe based reverse transcription PCR (qRT-PCR) was performed on swabs and tissues. Viral RNA was detected in rectal swabs on 2 and 4 DPI. One bat had viral RNA detected in rectal swab at 7 DPI, while the other was negative, and all additional time points were negative (figure 2.2). All oropharyngeal swabs were negative. Quantitative

RT-PCR was performed on lung, trachea, liver, small intestine, large intestine, spleen, and kidney collected 28 DPI. Viral RNA was detected in the lung of AJ-2p with a Ct value of 30.13. No viral RNA was detected in the other tissue samples. Results are qualitative, and not quantitative, as no tittered virus was available to generate a standard curve due to the lack of a cell culture system.

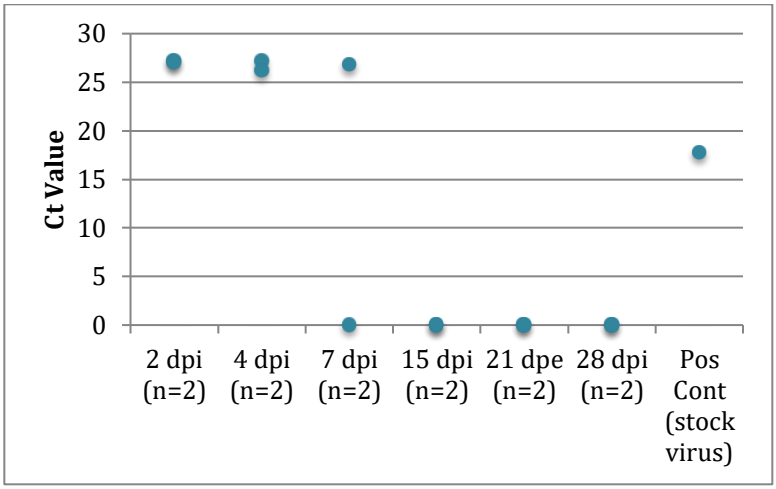

Figure 2.2: qRT-PCR Ct values on rectal swabs. Both bats had viral RNA detected in rectal swabs at 2 and 4 DPI. AJ-2p had viral RNA detected at 7 DPI, but not AJ-1p. Remaining time points had no viral RNA detected. Given that a cell culture system was not yet available to determine viral titers this data is qualitative and not quantitative.

For the time point study, probe based qRT-PCR was performed on rectal swabs, large intestines, small intestines and lung of both inoculated bats and transmission bats. Both transmission bat (AJ-t11 and AJ-t12) rectal swabs had viral RNA detected at 4 and 8 DPE. The other time points for AJ-t11 and AJ-t12 were negative (figure 2.3A). At 3 DPI ten rectal swabs were collected from all ten inoculated bats. Eight out of the ten inoculated bats had viral RNA detected at 3 DPI (AJ-h1, AJ-h2, AJ-h3, AJ-h4, AJ-h5, AJ-h6, AJ-h8, AJ-h9). At 6 DPI eight inoculated bats remained and rectal swabs were collected from each. All eight had viral RNA detected. At 10 DPI six inoculated bats remained and a rectal swab collected from each. Three out of the six remaining inoculated bats had viral RNA detected at 10 DPI (AJ-h7, AJ-h8, AJ-h9). All subsequent time points were negative (figure 2.3B).

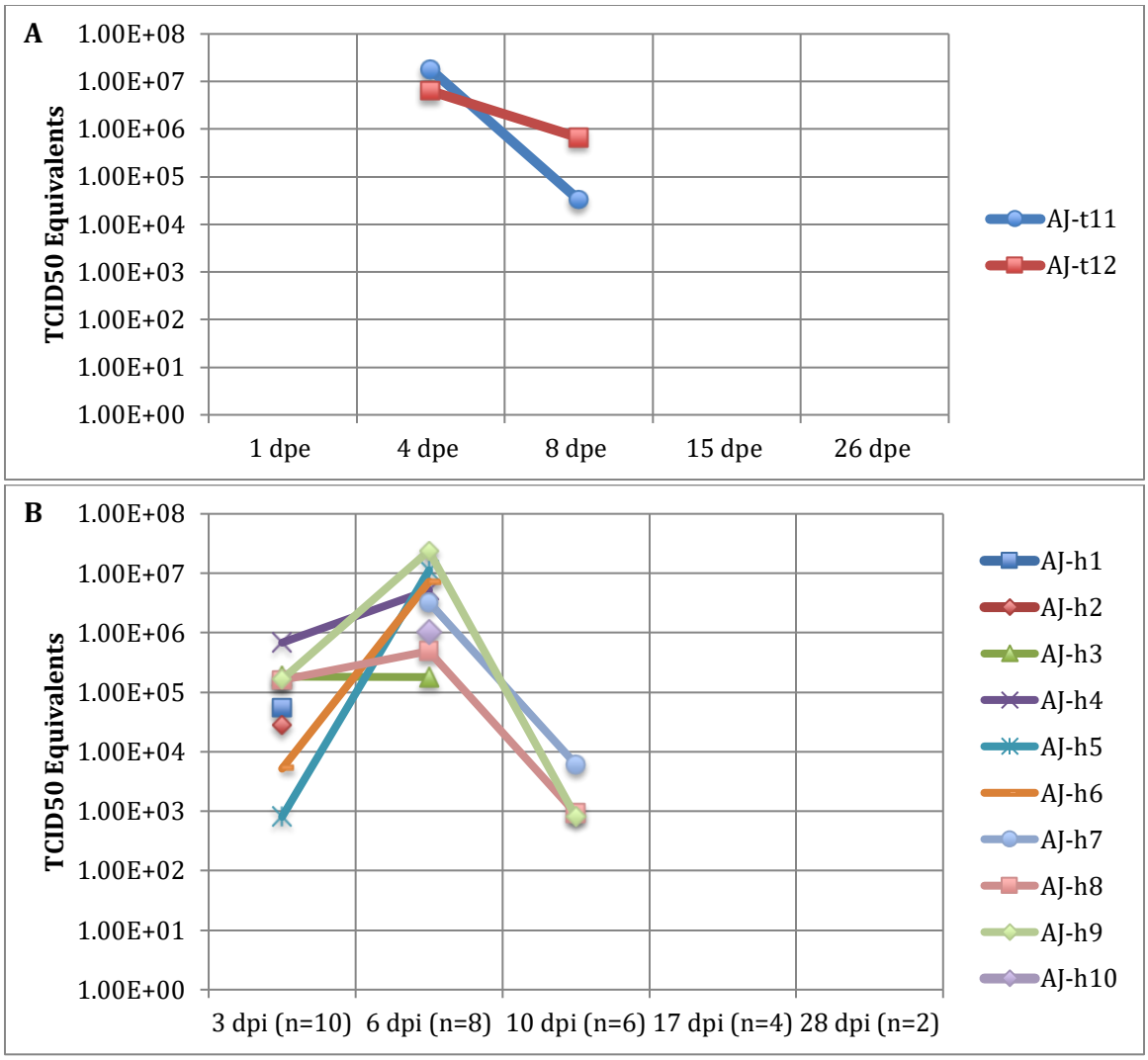

Figure 2.3: $TCID_{50}$ equivalents on rectal swabs from time-point study for transmission and inoculated bats. A) Both transmission bat (AJ-t11 and AJ-t12) rectal swabs were qRT-PCR positive for H18N11 at 4 and 8 DPE. The other time points for AJ-t11 and AJ-t12 were negative. B) Eight out of the ten inoculated bats were positive 3 DPI (AJ-h1, AJ-h2, AJ-h3, AJ-h4, AJ-h5, AJ-h6, AJ-h8, AJ-h9). Eight out of the eight inoculated bats were positive 6 DPI (AJ-h3, AJ-h4, AJ-h5, AJ-h6, AJ-h7, AJ-h8, AJ-h9, AJ-h10). Three out of the six inoculated bats were positive at 10 DPI (AJ-h7, AJ-h8, AJ-h9). All subsequent time points were negative.

Viral RNA was detected in large intestine with probe-based qRT-PCR at the following time points: AJ-h1 and AJ-h2 at 3 DPI, and Aj-h3 and AJ-h4 at 6 DPI. All other time points were negative in the large intestine (figure 2.4A). The small intestine had positive qRT-PCR values as follows: AJ-h1 at 3 DPI, AJ-h3 and AJ-4 at 6 DPI. All other time points were negative in the small intestine (figure 2.4B).

Quantitative RT-PCR on lung tissue yielded one positive at 6 DPI in AJ-h3. All other bat lung tissues were negative (figure 2.4C).

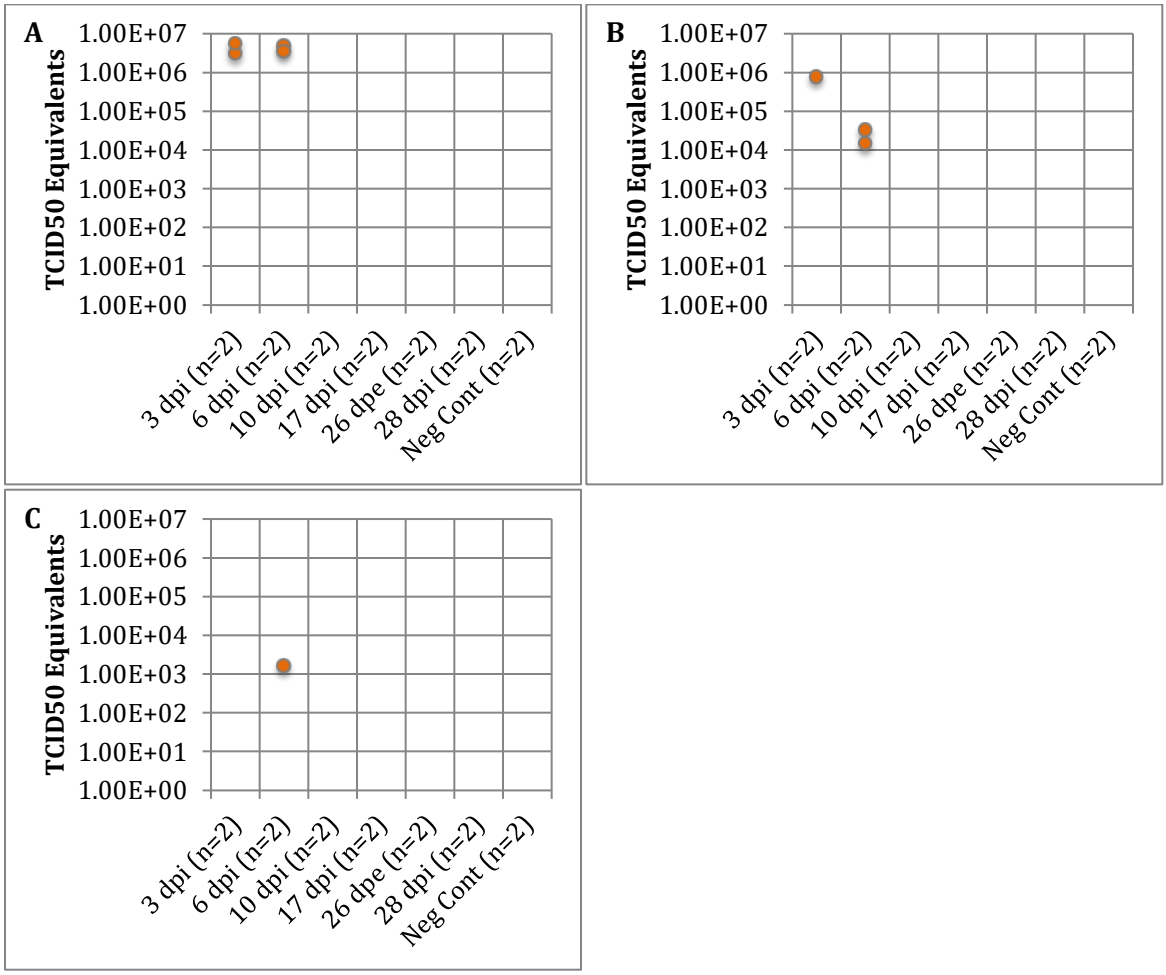

Figure 2.4: $TCID_{50}$ equivalents on large intestine, small intestine and lung from time-point study. A) Large intestine for AJ-h1 and AJ-h2 at 3 DPI, and AJ-h3 and AJh4 at 6 DPI were positive. B) Small intestine had positive values as follows: AJ-h1 at 3 DPI, AJ-h3 and AJ-4 at 6 DPI C) Lung tissue yielded one positive at 6 DPI in AJ-h3. All other bat lung tissues were negative.

2.3.5: Histology

Tissues cut in on bats to evaluate for histology included: heart, lung, liver, kidney, adrenal gland, spleen, intestine, and salivary gland. AJ-1p kidney had a focal area of mineralized infiltration in the inner medulla. The lungs had mild perivascular and peribronchial infiltrates and iatrogenic atelectasis. Intestines had a mild lymphocytic, plasmacytic and neutrophilic enteritis with crypt hyperplasia. Salivary

glands showed multifocal sialadenitis. All other tissues were within normal limits. AJ-2p lungs had multifocal histiocytic, lymphocytic interstitial pneumonia. There were variably dense aggregates of macrophages, lymphocytes and plasmacytes intermixed with fewer neutrophils that expand into the interstitium, especially around the adventitia with occasional accentuation of peribronchial lymphoid tissue. In some areas, on the pleural side, macrophages show increase phagocytic activity. Small intestines showed mild to moderate lymphocytic, plasmacytic and neutrophilic enteritis in which the lamina propria was expanded by cellular infiltrates of small to moderate numbers. Villous length was normal to mildly shortened. No lesions were observed in other tissues.

Tissues cut in to evaluate by histology were: heart, lung, liver, kidney, adrenal gland, spleen, intestine, and salivary gland. Tissues were graded based on extensiveness of lesion and annotated as no significant histological lesions, mild, moderate or severe. A grading of the histopathology lesions is listed in table 2.3.

Table 2.3: Graded histopathological lesions in time point study bats inoculated with H18N11 and transmission bats.

Days Post Infection/ Exposure	Bat ID	Sex	Heart	Lungs	Liver	Kidney	Adrenal gland	Spleen	Intestine	Salivary Gland
3 DPI	AJ-h1	Male	+	+	-	+	-	-	+	-
	AJ-h2	Female	+	++	-	-	-	-	+/++	+
6 DPI	AJ-h3	Female	-	+	+	-	-	-	+	-
	AJ-h4	Male	-	+	-	-	-	-	+	-
10 DPI	AJ-h5	Male	-	na	-	-	-	-	+/++	-
	AJ-h6	Female	-	na	-	-	-	-	++	-
17 DPI	AJ-h7	Male	+	-	+	+	-	-	-	-
	AJ-h8	Female	+	+	+	-	-	-	-	-
28 DPI	AJ-h9	Male	-	+	+	-	-	-	-	-
	AJ-h10	Female	-	+	-	+	-	-	-	-
26 DPE	AJ-t11	Male	-	-	-	+	-	-	-	-
	AJ-t12	Female	-	-	-	-	-	-	-	-
Neg Control	AJ-NCm	Male	+	+	-	-	-	-	-	-
	AJ-NCf	Female	+	+	-	+	-	-	-	++

Tissues were graded based on extent of lesion and annotated as (-) for no significant histological lesions, (+) mild, (++) moderate or (+++) severe, na denotes tissues not collected.

The negative control bats were kept in a cage for six days before being euthanasia. AJ-NCf had a focal area of fibrosis at the apex of the left ventricle of the heart. There was minimal vacuolation of individual cardiomyocytes and focal proliferation of endocardial endothelium with minimal infiltration of neutrophils (figure 2.5A). The outer medulla of the kidney contained multifocal areas of mineralization. The lungs showed multifocal alveolar hemorrhages with severe congestion. Hemorrhages occasionally extended to terminal bronchioles with which lumen contained detached epithelial cells (figure 2.5B). The salivary gland had multifocal periductular neutrophilic infiltrates intermixed with lymphocytes and macrophages which occasionally spilled into serous tubules and the periglandular adipose tissue (figure 2.5C). All other tissues were within normal limits. AJ-NCm heart had minimal focal segmental necrosis of cardiomyocytes with increased satellite cell activity that included a single mitotic figure. Lungs were moderately congested but did not show significant inflammation. No lesions were observed in other tissues.

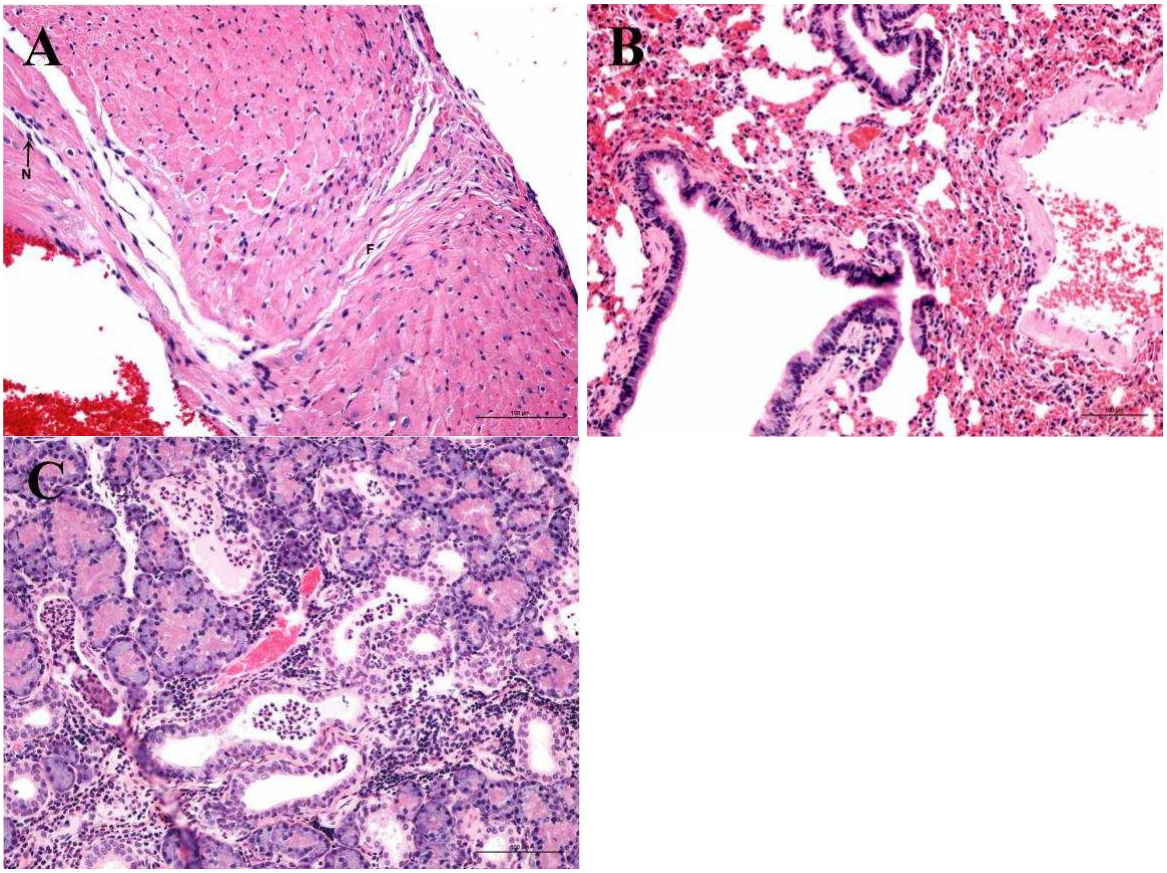

Figure 2.5: Histopathology (H&E) in the heart, salivary gland and lungs of negative control AJ-NCf
A) Heart with fibrosis (F) and focal proliferation of endocardial endothelium with minimal infiltration of neutrophils (arrow). B) Lung with moderate congestion. C) Sialadenitis.

For the time course study AJ-h1 at 3 DPE had lymphocytic interstitial myocarditis with multifocal individual cardiomyocyte degeneration. The kidney had a focal area of lymphocytic and plasmacytic infiltration in the renal pelvis. Lungs had multifocal microscopic alveolar hemorrhage and minimal perivascular lymphocytic interstitial pneumonia. Small intestine showed mild to moderate increases in proprial cellularity. No lesions were observed in other tissues. AJ-h2 at 3 DPE had multifocal interstitial myocardial infiltrates of primarily lymphocytes and fewer macrophages. The left ventricle showed multifocal areas of cardiomyocyte degeneration and necrosis with multifocal microscopic hemorrhages. The lungs had multifocal microscopic alveolar hemorrhage and minimal perivascular lymphocytic interstitial pneumonia. Similarly, to AJ-h1, the small intestine showed mild to

moderate increase in proprial cellularity (figure 2.6A). The salivary gland had multifocal lymphocytic interstitial sialadenitis. No lesions were observed in other tissues.

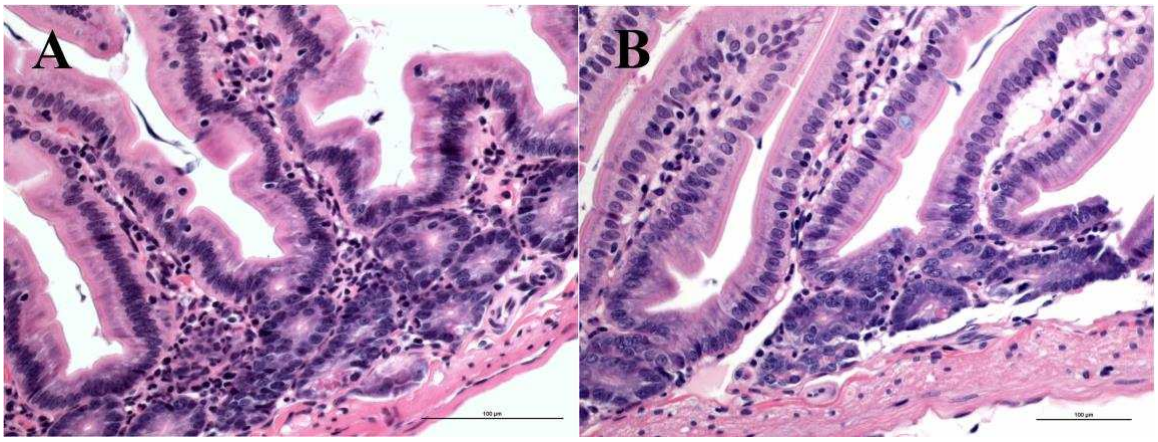

Figure 2.6: Small intestine of AJ-h2, 3 DPI with H18N11 compared to negative control bat (H&E). A) AJ-h2 small intestine with mild to moderate increase in proprial cellularity populated with lymphocytes and neutrophils. B) AJ-NCf negative control animal.

At 6 DPI AJ-h3 and AJ-h4 had similar lesions with similar severity in both the lungs and the gastrointestinal tract. In the lungs of both bats there was multifocal areas of alveolar hemorrhage with mild suppurative interstitial pneumonia. The small intestine of both bats showed mildly expanded lamina propria due predominately to lymphocytes and neutrophils. No lesions were observed in other tissues for these animals.

AJ-h5 and AJ-h6 at 10 DPI had blood clots inadvertently cut in as lung tissue for histology. Due to the severity of gross lung pathology it was difficult to distinguish between the two. As a result, no lungs were collected for histology on these two bats. Ten days post-inoculation time points will be repeated in another study. Histological lesions were seen in the gastrointestinal tract of both bats; plasmacytic and lymphocytic, neutrophilic enteritis (figure 2.7). Lesions in AJ-h6 were more severe than AJ-h5. No lesions were observed in other tissues.

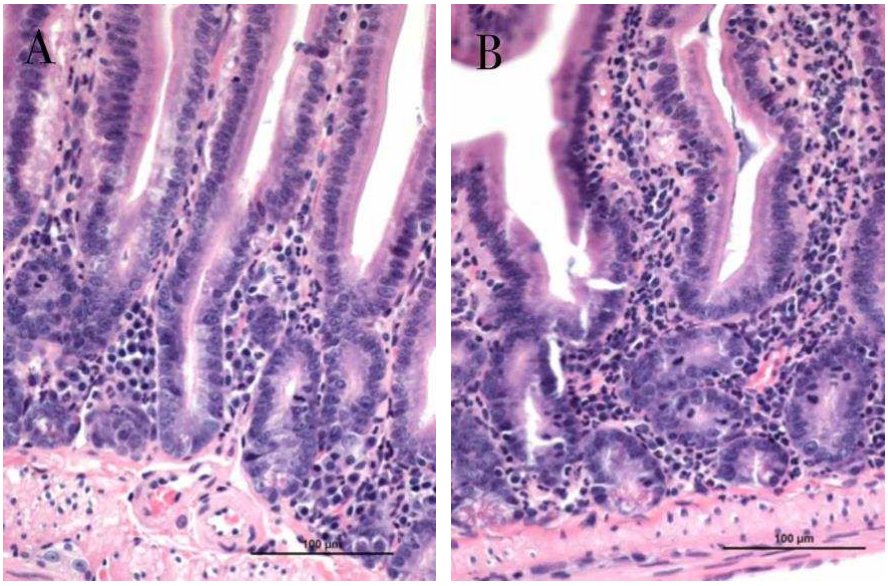

Figure 2.7: Small intestine of AJ-h5 and AJ-h6 at 10 DPI with H18N11 (H&E). A) AJ-h5 small intestine with mild to moderate increase in proprial cellularity populated with plasma cells and neutrophils. B) AJ-h6 shows greater infiltration of neutrophils and plasma cells, expanding both the lamina propria and the villi.

At 17 DPI, AJ-h7 heart showed increased satellite activity in the left ventricle. The liver had diffuse vacuolization of hepatocytes throughout the section. The outer and inner medulla of the kidney had moderate multifocal mineralization with no associated inflammation. AJ-h8 had myocardial interstitial infiltrates of lymphocytes and plasma cells. The liver showed random cellular infiltrates of neutrophils and lymphocytes. The lungs had interstitial septa slightly expanded by increased numbers of neutrophils and fewer macrophages and lymphocytes. No lesions were observed in other tissues.

At 28 DPI, AJ-h9 had vacuolar hepatopathy of the liver. Lung pathology was consistent with negative control animals. All other tissues were within normal limits. AJ-h10 kidney had minimal multifocal interstitial infiltrates of lymphocytes and plasmacytes with possible extramedullary hematopoiesis. Lung pathology was consistent with negative control animals.

The transmission bats, AJ-t11 and AJ-t12 were euthanized 26 days post exposure and had minimal histopathology. AJ-t11 had an inner medullary focus of mineralization of the kidney. AJ-t12 tissues had no lesions were observed.

2.3.6: Serology—ELISA

ELISAs were performed to assess for antibody against the H18N11 nucleoprotein. Bats euthanized at 3, 6 and 10 DPI did not seroconvert. Bats at 17 and 28 DPI and both transmission bats seroconverted with titers ranging from 200 to 3200 for antibody against nucleoprotein antigen (table 2.4).

Table 2.4: Immunoglobulin titers to H18N11 nucleoprotein antigen determined via ELISA. Seroconversion occurred at 17 DPI and 28 DPI as well as in transmission bats (AJ-t11 and AJ-t12).

Days Post Infection/Exposure	Animal ID	Titer
3 DPI	AJ-h1	<100
	AJ-h2	<100
6 DPI	AJ-h3	<100
	AJ-h4	<100
10 DPI	AJ-h5	<100
	AJ-h6	<100
17 DPI	**AJ-h7**	**400**
	AJ-h8	**200**
28 DPI	**AJ-h9**	**800**
	AJ-h10	**1600**
26 DPE	**AJ-t11**	**3200**
	AJ-t12	**1600**
NC	AJ-NCm	Neg
	AJ-NCf	Neg

2.4: Discussion

This study is the first to describe experimental infections of bats with the novel bat influenza viruses H17N10 and H18N11. This study provides insight into transmission, clinical disease, tissue tropism, pathophysiology, organ burden, and adaptive immunity.

Neither of the bat influenza viruses has been isolated from the wild (181, 182), and so viruses were rescued by reverse genetics. Reverse genetics uses cloned viral cDNA to generate virus. In influenza research, reverse genetics has allowed for genome mutation and recombination to better understand viral transmission, replication, virulence, and pathogenesis. It has application in vaccine development and anti-viral therapeutics (202). In the case of bat influenza viruses it allowed for the production of full-length genomes to better understand the viruses and virus-host interactions, given the virus has not been isolated from the wild (181, 182, 201). Because the viral strains used in this study were generated through reverse genetics from the published genome sequences, it is difficult to ascertain to what extent these viruses recapitulate natural, wild infection. However, our data is consistent with the findings from H18N11 discovery; viral RNA detected in rectal swabs and gastrointestinal tract in an *Artibeus* bat by qRT-PCR (182). Studies that used reporter expressing rescued influenza A viruses in animal models shows that pathophysiology and organ burden are consistent with wild-type viruses (203, 204). While it is difficult to know if the rescued H18N11 exactly emulates wild-virus infection, there is no reason to believe otherwise. Rescued influenza viruses are generally accepted in the scientific community (202, 205).

Jamaican fruit bats inoculated with H17N10 did not demonstrate apparent clinical disease, and virus was not detected in rectal or oropharyngeal swabs. Thus, Jamaican fruit bats likely do not support H17N10 infection. H17N10 was discovered in little yellow-shouldered bats of the genus *Sturnira*, family Phyllostomidae (181). Bats from the genus *Artibeus* also belong to the family Phyllostomidae, but being of different genera may make them genetically distinct enough as to not support H17N10 infection.

The pilot study H18N11 bats had no signs of clinical disease. In the time course study, mild disease was observed in the two transmission bats only in the form of mild ocular and nasal discharge, and epiphora in AJ-12t at 6 DPE, and AJ-11t and AJ-12t at 15 DPE. No signs of clinical disease were observed in earlier and later time points in transmission bats, nor in inoculated bats at any time point.

Lung pathology was seen in bats in the time course study at each time point up until 17 DPI. Lungs had varying degrees of congestion and cellular infiltrates (table 2.3). Given the severity of lung pathology in conjunction with the lack of clinical disease in inoculated bats, it seems as if these bats are very resistant to behavioral changes that may impart clinical disease. This type of stoicism is frequently seen in wildlife species, which may in part explain the lack of observable clinical disease.

Lung congestion was also observed in the negative control bats, which were housed for six days in the same type of cage as the inoculated and exposed bats (table 2.2). This would suggest that lung pathology is not solely the product of viral infection but there are other components altering normal physiology. These components may have influenced clinical disease presentation in the transmission bats. Stress, and prevention of flight and normal behaviors due to cage size may contribute to lung pathology. Additionally, study bats were kept in a separate room than colony bats. The room that housed study bats tended to have a decreased ambient temperature and less humidity. Taken with the qRT-PCR lung data, where only one bat, AJ-h3 at 6 DPI, was positive, lung pathology was most likely the product of environment and stress (figure 2.4C). Future studies will include altering bat housing to allow for free flight, increasing ambient temperature, and to minimize stress.

Pilot study H18N11 bats had positive rectal swabs between 2 and 7 DPI detected by probe-based qRT-PCR (figure 2.2). This was substantiated with the time course study where 8/10 bats at 3 DPI, 8/8 bats 6 DPI, and 3/6 bats at 10 DPI had viral RNA detected by qRT-PCR (figure 2.3B). Both transmission bats had viral RNA detected by qRT-PCR at 4 and 8 DPE (figure 2.3A). No viral RNA was detected by qRT-PCR in subsequent time points. Oral swabs were collected in the pilot study bats and had no viral RNA detected at any time point. This data provides evidence that H18N11 is shed in the feces, but not in saliva, and that infection of naïve animals may be through fecal-oral transmission. This data is also consistent with the original identification of the virus in rectal swabs, but not oral swabs of the flat-faced fruit bat in which the virus was first found (181, 182).

Further validating the H18N11 positive rectal swab results were qRT-PCR results of both large and small intestine from the time course study bats (figure 2.4A and B). Two/two bats had positive large intestine at 3 and 6 DPI for inoculated bats. One/two bats had positive small intestine at 3 DPI, and 2/2 positive at 6 DPI. This data indicates that H18N11 has a predilection for the gastrointestinal tract, and the entire length of the intestines is susceptible to viral infection. Furthermore, infection has a finite course, as viral RNA is detectable in early time points but not later time points. Consistent with this data was the gastrointestinal histopathology seen in the earlier time points of inoculated animals (3, 6, and 10 DPI). Small intestines at these time points showed varying degrees of cellular infiltration of neutrophils, lymphocytes or plasmacytes in the propria lamina (table 2.3, figures 2.6, 2.7). Similar lesions were seen in the pilot study H18N11 bats euthanized at 28 DPI.

The presence of viral RNA and histopathology in the gastrointestinal tract as well as PCR data that indicates fecal-oral transmission for H18N11 mimic influenza A infection in waterfowl and low pathogenic avian influenza (LPAI) in gallinaceous birds. In waterfowl influenza viruses are transmitted fecal-orally, there is a predilection for cells of the intestinal tract and no disease or histopathology associated with infection (103, 206, 207). It is the lack of disease with viral infection that produce the hypothesis that the virus and waterfowl have co-evolved over great periods of time, and water fowl serve as a reservoir and play a critical role in the natural history of the virus (103). LPAI have a high morbidity and low mortality in gallinaceous birds. Presentation may be subclinical to severe with chickens less vulnerable to disease than turkeys (208). Pathology may be seen in the upper and lower respiratory tract, reproductive tract, gastrointestinal tract, pancreas and kidneys (103, 208). In birds, IAV viruses can replicate in the gastrointestinal tract and cause related pathology. This is in contrast to influenza infection in mammals, which predominately causes a respiratory infection (209).

At and after 17 DPI all time course study bats seroconverted by ELISA, with titers ranging from 200 to ≥3200 (table 2.4). It was at 17 days when virus was no longer detected in the gastrointestinal tract

of bats, outlining the course of events of infection. Bats are most likely exposed fecal-orally to H18N11 and become infected. The virus has tropism for tissue of the gastrointestinal tract and is shed in feces. Between 10 and 17 days, bats begin to mount an adaptive immune response and clear the virus. A study that used H1N1 nucleoprotein as antigen detected antibody titers via ELISA in various species of animals post-vaccination. Antibody titers in pigs were approximately 1000 at 28 days post vaccinations. Foals, a month after parturition from vaccinated mares, had antibody titers between 100 and 1000 (210). When H18N11 was discovered, wild bat populations were screened for antibody titers using H18N11 HA and NA glycoproteins as antigen. Baseline dilution of serum was 1:1000 and antibody was detected in 55 out of 110 bats. Eleven of the 55 bats had titers ≥16,000 (181). Given these high titers, it may be warranted to extend our dilution series until signal extinction. There is evidence that bats have limited somatic hypermutation and affinity maturation, which generally account for low antibody titers in bats. (128, 211-217). Titers of ≥3200 in these experimentally infected bats, and ≥16,000 in wild bats are high compared to bat titers demonstrated against other viruses, and compared to the titers of pigs and foals; however, our investigation did not assess neutralizing antibody titers. Neutralizing titers are the product of SHM and affinity maturation.

Histological lesions were also present in the heart, liver, kidney and salivary gland of different animals (table 2.3, figure 2.5). These lesions were most likely incidental as similar lesions have been seen in other experiments that use Jamaican fruit bats (unpublished data) and were in the negative control bats. It is interesting to note that 7/14 bats, including both negative controls, had focal lesions in the heart (table 2.3). This species of bat may be prone to heart disease. These data are important to present because little is known of bat pathophysiology. As science continues to explore bat species, and we continue research in Jamaican fruit bats, background lesions need to be separated from lesions of interest. The more histopathology data presented, the more comparisons between species can be made and the more incidental lesions can be isolated from lesions of interest.

This study demonstrated that Jamaican fruit bats are susceptible to infection as indicated by viral RNA detected in rectal swabs and gastrointestinal tract of both inoculated bats and naïve bats exposed to inoculated animals. While histopathology was minimal, the $TCID_{50}$ equivalents were greater in tissues and rectal swabs compared to the inoculation dose, suggesting virus is propagating in the gastrointestinal tract. Does the pathophysiology of bat influenza viruses implicate bats as another potential reservoir for influenza viruses? True, tissue tropism in bats is unique from other animals and more closely resembles avian reservoir species infection. However, this is not substantial enough evidence to suggest bats are reservoirs for IAVs and there are no documented cases of H18N11 infection in people. Additionally, bat influenza viruses are highly divergent from other influenza A viruses—suggestive that bats and their influenza viruses have co-evolved for an extended period of time (181, 182). Given the limited tropism of the virus as demonstrated by cell culture experiments, and the failure of rescued H17N10 to be recovered from *Artibeus* bats—possibly due to the genetic diversity between Jamaican fruit bats and little yellow-shouldered bats, it is probable that bat influenza viruses are highly species specific and not a reservoir species (182, 189, 191, 194).

Studying these viruses could garner much information about the evolution of influenza viruses and a better understanding of bat viral ecology.

CHAPTER 3: EXPERIMENTAL INFECTION OF JAMAICAN FRUIT BATS (*ARTIBEUS JAMAICENSIS)* WITH ZIKA VIRUS

3.1: Introduction

Zika virus (ZIKV) was first isolated from a sentinel rhesus macaque in Uganda in 1947 and subsequently from *Aedes africanus* mosquitoes in the same location (218). The first human cases were identified in 1954 in Nigeria and serosurveys found evidence of a broad geographic distribution for ZIKV throughout Africa and Asia with sporadic cases of human disease (219, 220). The first recognized ZIKV epidemic occurred in Yap State, Federated State of Micronesia. An estimated 73% of residents were infected in 2007 and of those 18% had clinical disease (221). In 2013 a second epidemic occurred in French Polynesia with 28,000 individuals seeking medical care. During this outbreak, the incidence rate of Guillain-Barré syndrome (GBS) increased 20-fold and cases were temporally coupled to the ZIKV outbreak, establishing the first connection between GBS and ZIKV (222). The virus spread to Brazil were it was first diagnosed in 2015 (183, 184) and has since disseminated throughout much of South America, Central America, Caribbean, and the United States with more than 200,000 confirmed cases (223). ZIKV is a causal agent for Congenital Zika syndrome (CZS) and GBS and is therefore a virus of high concern (220).

The morbidity of ZIKV is approximately 20% with symptoms most frequently characterized by fever, maculopapular rash, conjunctivitis, arthralgia, malaise and headache (224). Taking into account the low morbidity, mild symptomology, and lack of epidemics, the virus was not considered a great threat until a correlation was made between ZIKV and neurological disease (224). CZS is a risk when a pregnant woman is infected and vertical transmission occurs. The mechanism of pathology is not well-delineated but infection of the central and peripheral nervous system of a fetus can result in severe microcephaly, collapsed skull, thin cerebral cortices with subcortical calcifications, ocular anomalies, and congenital contractures (225). ZIKV may also elicit GBS, a treatable autoimmune neuropathy brought on

by an infectious agent. GBS manifests as varying degrees of progressive weakness and flaccid paralysis (226).

Much has yet to be learned about the pathophysiology of ZIKV infection. Vectored through mosquitoes, predominately those of the *Aedes* genus, the virus is inoculated into the dermis and epidermis where it is capable of replicating in dermal fibroblasts, epidermal keratinocytes, and dendritic cells (227). In addition to a mosquito vector, ZIKV can be transmitted sexually, horizontally, and through blood transfusions (228, 229). Like other flaviviruses it seems ZIKV is able to use multiple cellular receptors to initiate cell entry including DC-SIGN, AXL, Tyro3, and TIM-1 (227). After point of entry, the virus may disseminate through the body via the blood. While tissue tropism has yet to be fully elucidated, RNA has been identified in urine, semen, blood, saliva, brain, placenta, and fetuses—suggesting broad tissue predilection and ubiquitously distributed receptors (230-233).

In the 1950s and 60s the susceptibility of bats to ZIKV was investigated. Shepherd and Williams, 1964 (234), screened 172 wild bats from 12 different species in Uganda for antibodies to ZIKV and found 16/44 Little free-tail bats (*Tadarida pumila*) and 26/36 Angolan free-tail bats (*T. condylura*) antibody positive to ZIKV by hemagluttination inhibition assay. Additionally, two Angolan free-tail bats were experimentally inoculated with ZIKV and serially bled. Both animals were viremic on days 2, 4 and 6—determined by 1:10 dilutions of serum that caused paralysis in mice (234). Simpson and O'Sullivan, 1968 (235), experimentally inoculated three straw colored fruit bats (*Eidolon helvum*), three Egyptian fruit bats (*Rousettus aegyptiacusi*), and five Angolan free-tail bats. Two of the straw colored fruit bats were viremic and seroconverted. One of the Egyptian fruit bats was viremic and two seroconverted. The Angolan free-tail bats were euthanized on days 1, 3, 5, 7 and 10 days post inoculation. At one day post infection a kidney was trace positive (235). Finally, Reagan et al (236) inoculated 20 little brown bats (*Myotis lucifigus*) by 5 different routes: intracranial, intraperitoneal, intradermal, intrarectal and intranasal. All groups, with the exception of the intranasal group, had neurological disease four to seven

days post inoculation. Brain tissue was positive in all animals with clinical disease, determined by inoculation of mice with brain suspension (236).

Considering the evidence that bats are naturally susceptible to ZIKV and that little brown bats develop disease, could bats serve as a natural reservoir host for ZIKV in the New World? To test this hypothesis, we inoculated Jamaican fruit bats (*Artibeus jamaicensis*), one of the most common bats in the Caribbean, Central America and Mexico, with ZIKV to examine the virology, immunology and pathology of infection. Although virus was detected in several organs, including the testes of males and brains, no signs of clinical disease were found and little evidence of viremia or viruria. These results suggest that Jamaican fruit bats are not likely a reservoir host but that ZIKV infection may constitute a wildlife disease threat to bats.

3.2: Methods and Materials

3.2.1: Bats

All animal procedures were approved by the Colorado State University (CSU) Institutional Animal Care and Use Committees and were in compliance with U.S. Animal Welfare Act. CSU has a captive colony of Jamaican fruit bats (*Artibeus jamaicensis*), a neotropical fruit bat indigenous to much of South America, Central America and the Caribbean (237). Colony bats are kept as described in chapter 2, section 2.2.1.

For infection experiments, bats were trapped using a butterfly net and placed in an 20" d x 12" w x 18" h cage for 24 hours prior to inoculations to allow for acclimation. Hanging clothes were provided for roosting and coverage. Food and water are placed in open trays in the bottom of the cage and changed daily. Tray liners were changed every two days, and cages and hanging clothes are changed every two weeks. Due to the social nature of these bats, minimums of two bats were kept in cages at all times to mitigate potential stress.

3.2.2: Experimental infection

Two sets of experiments were performed; a pilot study and a time course study. Zika virus strain PRVABC59 was used for both studies. PRVABC59 was isolated in 2015 by Centers for Disease Control and Prevention (Fort Collins, CO) from an infected individual who traveled to Puerto Rico (GenBank accession no. HQ234499). The virus stock was prepared by inoculating Vero E6 cells and collecting supernatant five days later. The titer is $3x10^7$ plaque forming units (p.f.u.) per ml of media.

For the pilot study, three male bats were anesthetized with 1% to 3% isoflurane to effect with an oxygen flow rate of 1.5 L/min, administered with a gas mask. Animals were placed on a heating pad to maintain body temperature and respirations continuously monitored. The dorsum of each animal was disinfected with 70% ethanol and 25ul containing $7.5x10^5$ p.f.u of virus was administered subcutaneously (sc) at the level of the scapula with a sterile hypodermic 25 gauge needle in a biosafety cabinet. When procedures were finished, bats were removed from isoflurane and placed back in the cage in ventral recumbency. Respirations were monitored until animal was fully awake and ambulated normally. Bats were identified as AJ-z7, AJ-z8 and AJ-z9. Animals were euthanized at 28 days post-inoculation (DPI).

For the time course study, six male bats were anesthetized under the same protocol as the pilot study. Animals were placed in ventral recumbency. After disinfecting the dorsum of each animal with 70% ethanol, 0.15mls of 1% lidocaine was administered sc. at the level of the last rib with a 25 gauge sterile hypodermic needle as a local anesthetic. IPTT300 transponders (BioMedic Data Systems, Inc., Seaford, DE) were inserted sc. at the level of the caudal edge of the scapula. Twenty-five microliters containing $7.5x10^5$ p.f.u of virus was administered sc at the level of the cranial edge of the scapula. Recovery followed the same protocol as for the pilot study bats. Animals were identified as AJ-z1 through AJ-z6. AJ-z1 and AJ-z2 were euthanized at two DPI. AJ-z3 and AJ-z4 were euthanized at 5 DPI. AJ-z5 and AJ-z6 were euthanized at 10 DPI.

Female bats were excluded from the study because they are prioritized for breeding to sustain and expand upon the colony.

3.2.3: Monitoring

For the pilot study, bats were visually assessed twice daily for fourteen days, and then assessed once a day for an additional fourteen days. For the time course study, bats were assessed twice a day throughout the experiment. For both studies behavior, ability to ambulate, respirations, presence of oral or nasal discharge, and fecal consistency were all assessed.

3.2.4: Urine Collection

During the time course study urine was collected at 2, 3, 5 and 10 DPI from as many bats as possible. Urine was collected by allowing bats to grasp screen cloth with their feet and then the bat was placed in a clear solo cup (Dart Container, Lake Forest, IL) with the screen covering the top of the cup as a lid, and kept in place with a rubber band. This would allow the bats to hang in a clear container. Bats were monitored for 45 minutes. If they urinated, bats were removed from the collection cups and placed back in the cage without disrupting the urine. Urine collection was attempted on all remaining bats at each time point, but not all bats would urinate at each collection attempt. Urine was successfully collected as follows: two DPI from AJ-z3 and AJ-z4; three DPI from AJ-z3, AJ-z5 and AJ-z6; five DPI from AJ-z3, AJ-z4, AJ-z5 and AJ-z6; and ten DPI from AJ-z5 and AJ-z6. Urine was pipetted off the surface of the cup with a sterile pipette tip and put in a 1.5 ml microcentrifuge tube and stored at -80°C for future use. Urine volume ranged between 5 ul and 15 ul.

3.2.5: Euthanasia, Blood Collection and Necropsy

Bats were deeply anesthetized and maintained with 3% isoflurane and an oxygen flow rate of 1.5 L/min. Deep pain was assessed by firmly pinching skin and toes with forceps and assessed for any response. A thoracotomy was then performed with sterile standard scissors to puncture through the skin,

muscle and diaphragm just caudal to the sternum and cut through the wall of the chest cavity caudally to cranially.

Cardiac blood was collected with a 21 gauge sterile needle inserted into the apex of the heart. A maximum blood volume of between 1 and 1.5 ml is collected in a syringe and transferred to a red top tube (RTT). RTTs sat at room temperature for one hour to allow a clot to form and then centrifuged at 1000 x g for 10 min at room temperature. Serum was removed from the clot, placed in a new microcentrifuge tube and stored at -20°C.

Serum from bats at 2 and 5 DPI were used to assess for viremia. Serum from 10 DPI and the 28 DPI pilot study bats were used to determine antibody titers. Because blood draws yield a small volume of blood it was necessary to prioritize samples to optimize data retrieved.

Necropsies were performed immediately after euthanasia. Bats were assessed for gross pathology. The following tissues were collected for both experiments: heart, lung, liver, spleen, kidney, urinary bladder, prostate, testes, and brain. A portion of tissues were collected and kept at -80°C for RNA extraction, and a portion placed in 10% buffered formalin for histology.

For a negative control animal, a male bat was trapped from the colony and euthanized under the same protocol as the experimental infection bats.

3.2.6: Serology—ELISA

Vero E6 cells were propagated to 60% confluency in a 96-well tissue culture plate and infected with ZIKV strain PRVABC at an m.o.i. of 0.1. After a one hour incubation period, virus was removed and replaced with 2% FBS-DMEM and incubated for a maximum of three days. Media was then replaced with 85% acetone for 20 minutes at -20°C to fix virus-infected cells to plate and serve as an antigen for

enzyme-linked-immunosorbent assay (ELISA). Plates were stored at 4°C until use and used within two weeks. Plates were washed five times with PBS containing 0.05% Tween 20 and blocked with SuperBlock T20 (TBS) Blocking Buffer (Thermo Fisher Scientific, Waltham, MA) for one hour at room temperature. Serum from an uninfected bat was used for a negative control. A convalescent human serum sample was used as a positive control. A two-fold serial dilution was used starting at 1:100 to 1:12800. Diluted serum was placed in wells and incubated for two hours at room temperature. Serum was removed and plates washed. HRP-conjugated protein A/G (Thermo Fisher Scientific, Waltham, MA) was added at a concentration of 2 ug/ml to each well, and incubated for 30 minutes at room temperature. HRP-conjugated protein A/G was used in place of a secondary antibody as it targets the Fc portion of an antibody, which is highly conserved and therefore can be used for multiple animal species (238). Plates were washed and 150 ul of ABTS Peroxidase Substrate (2 component) (KPL, Gaithersburg, MD) added according to manufacturer's instructions, incubated at room temperature for 30 minutes, and then 150 ul of ABTS Peroxidase Stop solution (KPL, Gaithersburg, MD) added. Plates were read on a EMax Plus Microplate Reader (Cambridge Scientific, Watertown, MA). Absorbance was measured at 405 nm and the limit of detectable response was set at three standard deviation values above mean negative control serum.

3.2.7: RNA Extraction

TRIzol Reagent was used according to Ambion, Life Technologies protocol for RNA extraction from serum-cell supernatants, serum, urine and tissues. For tissues, approximately 50 mg of tissue was homogenized with one mL of TRIzol Reagent. A 5mm stainless steel bead (Qiagen, Valencia, CA) was used with a TissueLyser LT (Qiagen, Valencia, CA) at 50 Hz for 5 minutes. One ml of TRIzol was added to urine to 5 to 15 ul of urine. One ml of TRIzol was added to 160 ul of serum from AJ-z2, AJ-z3, and AJ-z4. Two-hundred microliters of serum-cell supernatants were added to one ml of TRIzol. Samples were then incubated at room temperature for 5 minutes. Chloroform (Thermo Fisher Scientific, Waltham, MA) was added, samples were mixed, incubated for 3 minutes at room temperature and centrifuged at

12,000 x g for 15 minutes at 4°C. The aqueous phase was removed, 4 ug of glycogen (Thermo Fisher Scientific, Waltham, MA) and 100% molecular grade isopropanol added (Thermo Fisher Scientific, Waltham, MA). Samples were incubated at room temperature for 10 minutes and then centrifuged at 12,000 x g for 10 minutes at 4°C. Supernatant was removed and 75% molecular grade ethanol (Thermo Fisher Scientific, Waltham, MA) was added to RNA pellet. Samples were vortexed and centrifuged at 7500 x g for 5 minutes at 4°C. Wash was removed and air-dried. RNA was re-suspended in RNase-free water and stored at -80°C for future use.

3.2.8: Viral RNA Detection in Serum Samples

Vero cells were grown to 70 to 80% confluency in a 48-well tissue culture plate with 10% FBS-DMEM. Media was removed and 100 ul of bat serum from 2 DPI bats and 5 DPI bats was inoculated onto cells. Additionally, serum from each bat was diluted 10-fold in 2% FBS-PBS supplemented with 1% calcium and magnesium, and inoculated onto cells. Samples were incubated for one hour at 37°C. Inoculum was removed and cells washed twice in sterile PBS. Two-percent FBS-DMEM was added to wells and plates were incubated at 37°C, 5% CO_2. Cells were assessed daily for cytopathology (CPE) thru day 7. Two-hundred microliters of the supernatant was removed on day 7 and used for RNA extractions. An additional 100 ul of supernatant was blind passaged onto Vero cells at 70 to 80% confluency. Cells were incubated for one hour at 37°C, washed twice with sterile PBS and 2% FBS-DMEM added. Cells were monitored daily for CPE for seven days, of which there was none. On day seven, supernatant was removed and TRIzol extractions performed for RNA recovery. Serum was treated as such in an attempt to amplify viral load and increase assay sensitivity serum may not be the most sensitive diagnostic sample (239-242).

If any serum was remaining it was directly used for TRIzol RNA extractions. Serum samples remained from AJ-z2 at 2 DPI, and AJ-z3 and AJ-z4 at 5 DPI. No serum remained from AJ-z1.

3.2.9: qRT-PCR

Roche Real Time Ready RNA Virus Master Kit (Roche, Indianapolis, IN) was used on RNA extracted from serum-cell supernatants, serum, urine and tissue to assay for ZIKV RNA according to manufacturer's instructions. Primers used were ZIKV 1086 (CCGCTGCCCAACACAAG) and ZIKV 1162c (CCACTAACGTTCTTTTGCAGACAT). Probe was ZIKV 1107-FAM (AGCCTACCTTGACAAGCAGTCAGACACTCAA) (243). Two-hundred nanograms of sample RNA was added to each reaction. Reactions were performed in duplicate. Standards were generously provided by Dr. Rushika Perera, Arthropod-Borne Infectious Diseases Laboratory, Department of Microbiology, Immunology and Pathology, Colorado State University. Standards were a non-infectious clone of full length ZIKV strain PRVABC59 by which concentration was determined through optical density. Molecular weight of the genome sequence was used to calculate copy number (96). A $\log_{10}$ dilution series of the standard was made and linear regression used to determine copy number equivalents of positive samples. A light-cycler 96 (Roche Diagnostics Corporation, Indianapolis, IN) was used. Amplification was performed according to manufacturer's protocol for Roche Real Time Ready RNA Virus Master Kit (Roche Diagnostics Corporation, Indianapolis, IN) with PCR conditions as follows: 8 min at 50°C, 30 s at 95°C, and 45 cycles of 10 s at 95°C, 20 s at 60°C and 10 s at 72°C.

3.2.10: Histology

Tissues fixed in 10% buffered formalin were cut in and submitted to Colorado State University Veterinary Diagnostic Laboratory (CSU VDL, Fort Collins, CO) for paraffin embedding, sectioning and staining with hematoxylin and eosin, as well as immunohistochemistry (IHC). Tissues cut in on bats to assess for histology included: heart, lung, liver, kidney, testes, prostate, urinary bladder and brain. Additionally, for AJ-z3 and AJ-z5 mandibular salivary gland was cut in. AJ-z4 had esophagus and lymphoid tissue that included palatine salivary gland cut in. Antibody for IHC was a polyclonal rabbit antibody that targets preM and E proteins of ZIKV and was provided by CSU VDL's pathology

department. The Bond-III automated instrument (Leica Biosystems, Wetzlar, Germany) was used for IHC staining.

3.3: Results

3.3.1: Experimental Infections

Bats for this project were taken from the Colorado State University breeding colony. Two animal-infection experiments were conducted; a pilot study and a time course study. In the pilot study three male bats (AJ-z7, AJ-z8, AJ-z9) were experimentally inoculated with $7.5x10^5$ plaque forming units (p.f.u) ZIKV, strain PRVABC59. Bats were monitored for 28 days for signs of clinical disease, then euthanized for necropsy and sample collection. After demonstration of susceptibility in the pilot study, a time course study was conducted. Six male bats (AJ-z1 through AJ-z6) were identically inoculated and two were euthanized at 2, 5 and 10 DPI.

Bats were visually assessed twice a day for signs of disease as we previously found for Tacaribe virus infection (215), including lethargy and neurological abnormalities, unresponsiveness to stimuli, behavioral abnormalities (i.e. not roosting in a group), tremors, ataxia, and inability to fly; however, no conspicuous signs of disease were observed. Necropsies immediately followed euthanasia and no gross pathology was observed. Necropsies immediately followed euthanasia and gross pathology was assessed at time of organ collection. No apparent gross pathology was seen on necropsy in any animal.

3.3.2: Serology—ELISA

ELISA was performed on two-fold serial dilutions of serum from 1:100 to 1:12800. Negative control serum was from an uninfected colony bat. Positive control serum was a convalescent human sample. The three pilot experiment bats (AJ-z7, AJ-z8, AJ-z9) euthanized at 28 DPI each had antibody titers of 3200. The positive control titer was 12800 and the negative control was negative (<100) (table 3.1).

Table 3.1: Individual antibody titers in 28 DPI bats inoculated with ZIKV by ELISA.

Animal ID	Titer
AJ-z7	3200
AJ-z8	3200
AJ-z9	3200
Pos Control (convalescent human sample)	≥12800
Neg Control (uninfected bat)	Neg

3.3.3: qRT-PCR

Quantitative probe based reverse transcription PCR (qRT-PCR) was performed on serum-cell supernatants, serum, brain, lung, liver, spleen, kidney, urinary bladder, prostate and testes for bats from both studies. In addition, urine collected during the time course study was assayed. Viral RNA copy numbers were estimated using a non-infectious clone of full length ZIKV strain PRVABC59 with concentration determined through optical density (96). Urine from AJ-z6 at 3 DPI had $5.32x10^2$ copy number equivalents and AJ-z7 at 5 DPI had $3.52x10^2$ copy number equivalents. AJ-z1, euthanized at 2 dpi, had a positive brain by qRT-PCR with $1.08x10^3$ copy number equivalents. All other samples were negative. Serum samples from early infection bats (AJ-z2, AJ-z3 and AJ-z4) were negative. Sera were blind passaged on Vero E6 cells and all were negative.

3.3.4: Histology

3.3.4.1: H&E

Tissues cut in on bats to assess for histology included: heart, lung, liver, kidney, testes, prostate, urinary bladder, and brain on all animals, and salivary gland on 3/9 animals. A summary of the consistent histopathology findings is listed in table 3.2 at the end of this section.

For the time course study AJ-z1 at 2 DPI had congested lungs with multifocal areas of interstitial pneumonia, mild intraalveolar hemorrhages and mild collapse. Terminal airways had slightly increased

amounts of mucous. Kidneys had multifocal interstitial infiltrates of small numbers of lymphocytes. All other tissues were within normal limits. AJ-z2 at 2 DPI lungs showed milder pathology than AJ-z1 with minimal interstitial to perivascular infiltrates predominately lymphocytes and macrophages with a band of collapsed air spaces parallel to the pleural surface. There were focal lesions in the left ventricle of the heart where there was individual cell loss or fragmentation of the sarcoplasm and infiltration of small numbers of macrophages, lymphocytes and satellite cells. No lesions were observed in other tissues.

AJ-z3 at 5 DPI lungs had minimal focal interstitial hystiocytic pneumonia with atelectasis. Kidneys showed multifocal chronic lymphocytic, histiocytic pyelitis of the renal pelvis. Mandibular salivary gland showed focal moderate cellular infiltrates of lymphocytes and macrophages that surrounded ducts, and contained detached and degenerate epithelial cells. Rare apoptosis was evident in the lining epithelium of ducts. No lesions were observed in other tissues. AJ-z4 at 5 DPI had lungs with minimal alveolar septal infiltrates scattered within collapsed lung parenchyma with multifocal microscopic hemorrhages. Kidneys had multifocal areas of mineralization. In the outer medulla and at the corticomedullary junction were rare perivascular infiltrates with lymphocytes and plasmacytes. Esophagus and lymphoid tissue was cut in with palatine salivary gland that showed focal lymphocytic and plasmacytic inflammation. Moderate numbers of lymphocytes and plasma cells were arranged in columns parallel to the respiratory mucosal epithelium of the nasopharynx. The lumen contained increased amounts of mucous and a few inflammatory cells, mainly neutrophils and lymphocytes. There was focal testicular degeneration manifested by presence of giant spermatids in the lumen of affected seminiferous tubules and accumulation of a small numbers of lymphocytes. No lesions were observed in other tissues.

AJ-z5 at 10 DPI lungs had minimal interstitial to perivascular infiltrates with multifocal atelectasis and microscopic hemorrhages. The left papillary muscle of the heart showed rare areas of multifocal cardiomyocyte necrosis characterized by rounding up of individual cardiomyocytes which

appeared hypereosinophilic. There was loss of cross striations, and minimal interstitial hypercellularity due to increased activity of satellite cells and infiltration of small numbers of lymphocytes. Kidneys had an area of focal lymphocytic, plasmacytic pyelitis. Additionally, there was a focal area of mineralization and inflammation in the inner medulla. Mandibular salivary glands were cut in but within normal limits. All other tissues were within normal limits. AJ-z6 at 10 DPI had occasional focal inflammation and cardiomyocyte degeneration in the left ventricle and septum of the heart. Area A3 of the hippocampus in the brain showed focal pyramidal neuronal necrosis with a focal area of mineralization around a vessel in the cerebral cortex and focal gliosis (figure 3.1). No lesions were observed in other tissues.

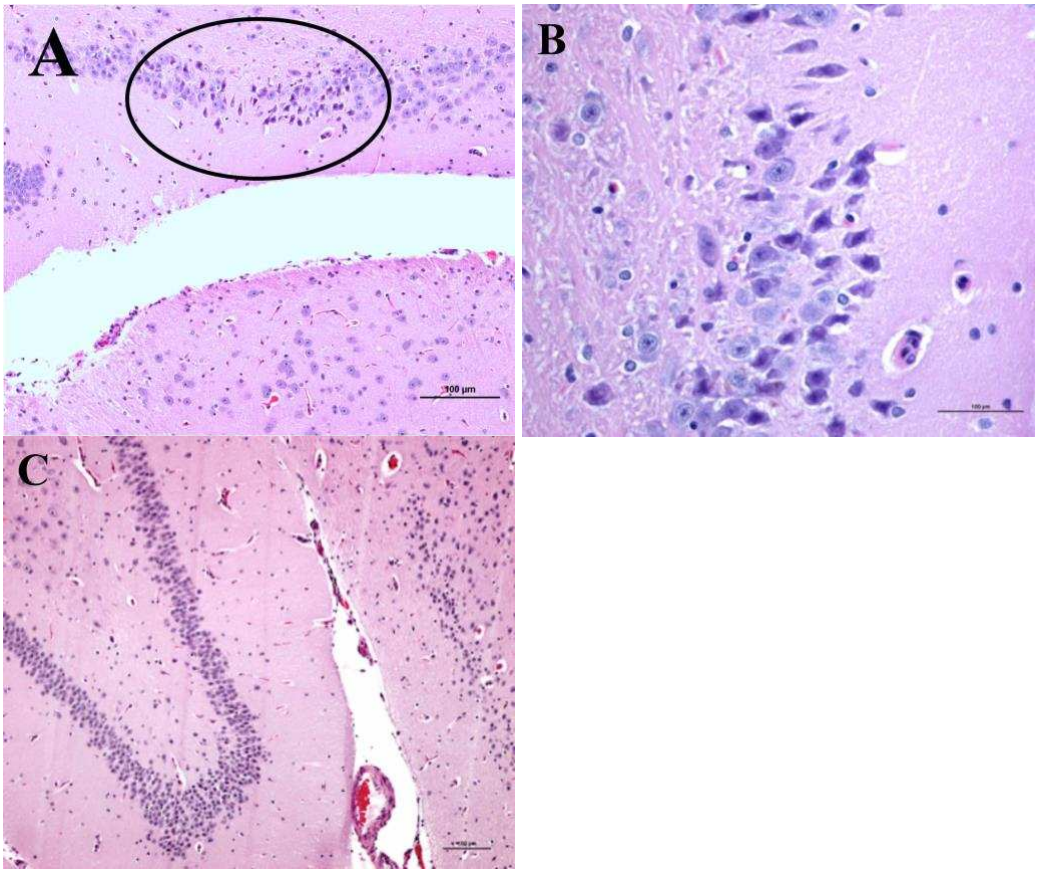

Figure 3.1: Hippocampus from AJ-z6, 10 DPI compared to negative control bat (H&E). A) AJ-z6, 10 DPI area A3 of the hippocampus with focal pyramidal neuronal necrosis, circled. B) AJ-z6, 10 DPI 400x magnification of lesion from figure A demonstrating angular, pyknotic, hypereosinophilic nuclei of necrosed neuronal cell bodies. C) Negative control bat hippocampus showing normal focal pyramidal neuronal cell bodies.

In the pilot study bats, AJ-z7 at 28 DPI had more prominent interstitial pneumonia with congestion of the lungs compared to earlier time points. The heart had minimal cardiomyocyte degeneration and necrosis with hyper cellular interstitium and increased amounts of mature fibrous connective tissue. The kidney had focal interstitial infiltrates of the renal interstitium. The brain showed degenerate neurons in area A3 of the hippocampus. All other tissues were within normal limits. AJ-z8 had minimal focal testicular degeneration (figure 3.2). No lesions were observed in other tissues. AJ-z9 had perivascular lymphocyte infiltrates and atelectasis. Heart demonstrated locally extensive lymphocytic and histiocytic pericarditis. Kidney showed multifocal interstitial lymphocytic infiltrates. Brain had focal lymphocytic, perivascular infiltrates of small numbers at the subfornical commissure. The reticular formation showed multifocal neuronal degeneration.

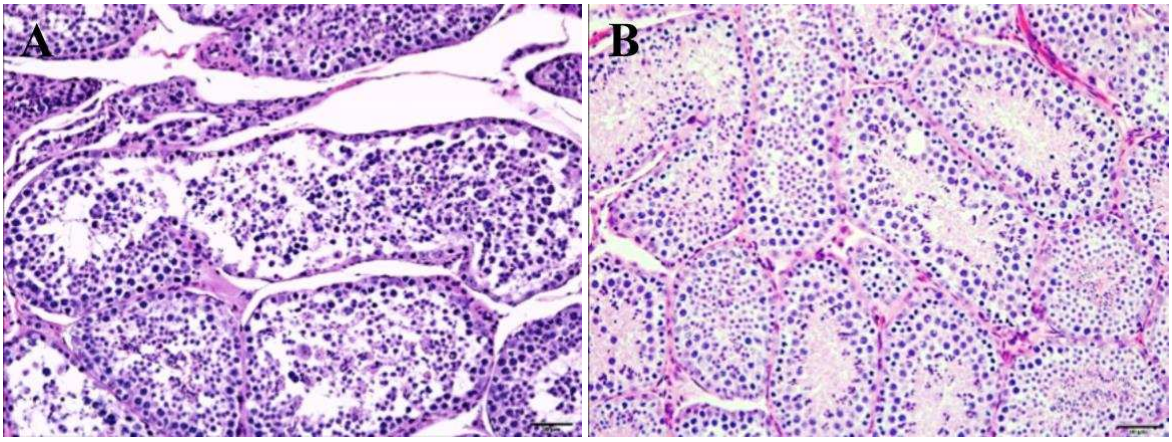

Figure 3.2: Testes from AJ-z8, 28 DPI compared to negative control bat (H&E). A) Focal testicular degeneration. Seminiferous tubules are disorganized with no mature sperm in lumen and giant spermatids. B) Negative control bat normal testes.

Table 3.2: Histopathological findings in bats inoculated with ZIKV.

		2 d.p.i.		5 d.p.i.		10 d.p.i.		28 d.p.i.			Total
		AJ-z1	AJ-z2	AJ-z3	AJ-z4	AJ-z5	AJ-z6	AJ-z7	AJ-z8	AJ-z9	
Lungs	Pneumonia	+/-	+/-	+/-				+/-		+/-	5/9
	Infiltrates and microscopic hemorrhage				+/-	+/-					2/9
Heart	Cellular infiltrates		+/-								1/9
	Cardiomyocyte necrosis					+/-	+/-	+/-		+/-	4/9
Kidney	Pyelitis			+/-		+/-					2/9
	Cellular infiltrates	+/-			+/-			+/-			3/9
Testes	Degeneration and lymphocyte infiltration				x				x		2/9
Brain	Neuronal degeneration						x	x		x	3/9
Sal. Gl.	Sialoadenitis			x							1/3

Lesions in the lungs, heart, and kidney may be incidental as similar lesions have been seen in other experiments with different infectious agents (unpublished data), indicated by "+/-". Lesions in the testes, brain and salivary gland (sal. gl.) may be due to ZIKV infection, indicated by an "x".

3.3.4.2: Immunohistochemistry

Tissues were stained with an antibody for ZIKV by CSU Experimental Pathology Core. AJz-3 at 5 DPI had inflammation of the mandibular salivary gland with immunoreactivity (figure 3.3). AJ-z5 at 10 DPI had immunoreactive cells in the brain and mononuclear cell immunoreactivity in the testes (figure 3.4). Additionally, AJ-z5 demonstrated immunoreactivity in purkinje cells of the cerebellum (figure 3.5A). AJ-z8 at 28 DPI had immunoreactive cells around the pulmonary arteries in the lungs (figure 3.6A). AJ-z8 also had perivascular immunoreactivity in the tunica albuginea of the testes (figure 3.7A). Scrotal skin was also cut in and had focal lymphocytic dermatitis with immunoreactive mononuclear cells (figure 3.7D). Cell morphology consistently identified mononuclear cells, macrophages and fibroblasts as the cell types that were immunoreactive with ZIKV antigen.

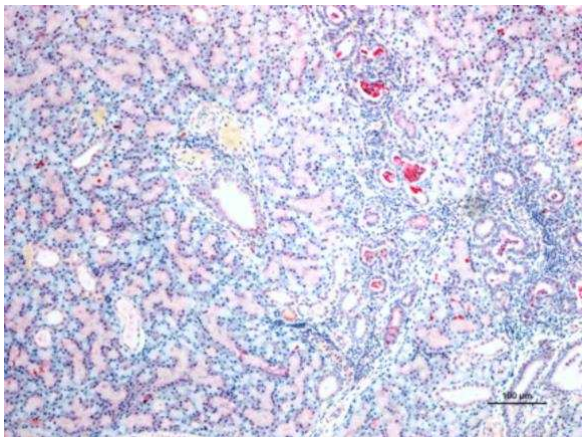

Figure 3.3: Salivary gland from AJ-z3, 5 DPI, IHC staining for ZIKV antigen. Glandular immunoreactivity of the salivary gland.

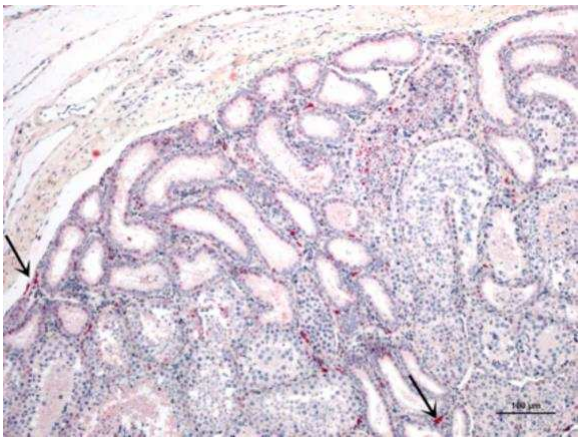

Figure 3.4: Testes from AJ-z5, 10 DPI, IHC staining for ZIKV antigen. Mononuclear cell immunoreactivity, arrows.

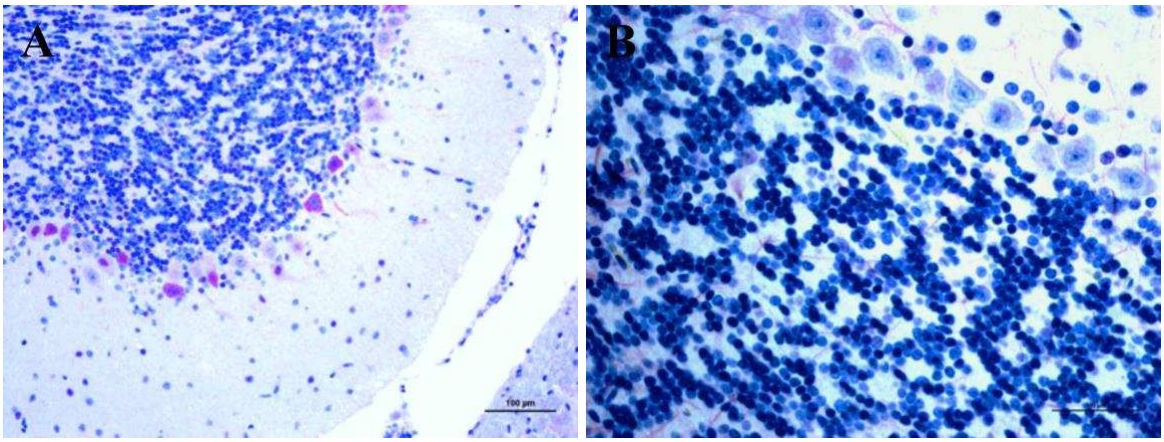

Figure 3.5: Immunoreactivity in AJ-z5, 10 DPI cerebellum, IHC staining for ZIKV antigen compared to negative control bat. A) Multifocal purkinje cell immunoreactivity in AJ-z5. B) Negative control cerebellum.

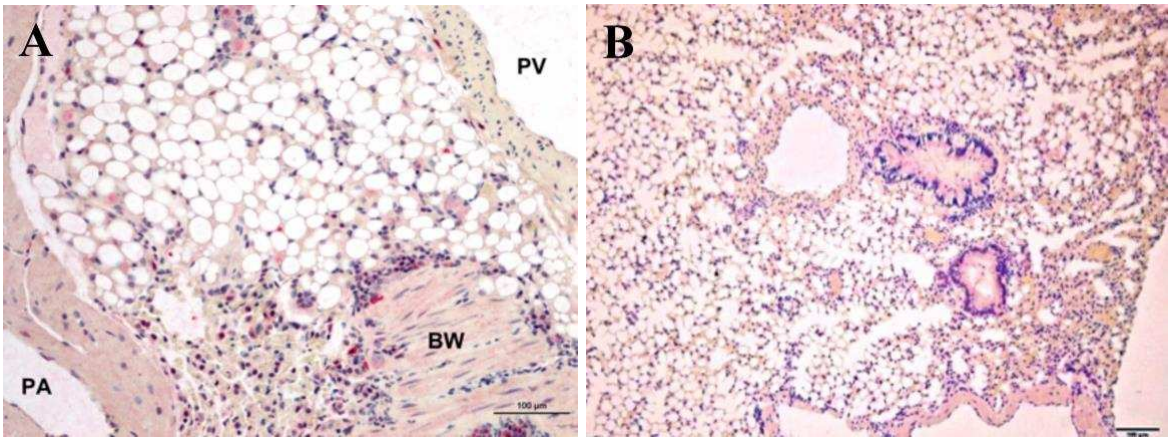

Figure 3.6: Immunoreactivity in AJ-z8, 28 DPI lung, IHC staining for ZIKV antigen compared to negative control bat. A) Hilum of the lung shows immunoreactivity in mononuclear cells around the pulmonary artery. PA, pulmonary artery. PV, pulmonary vein. BW, bronchiolar wall. B) Negative control without immunoreactivity.

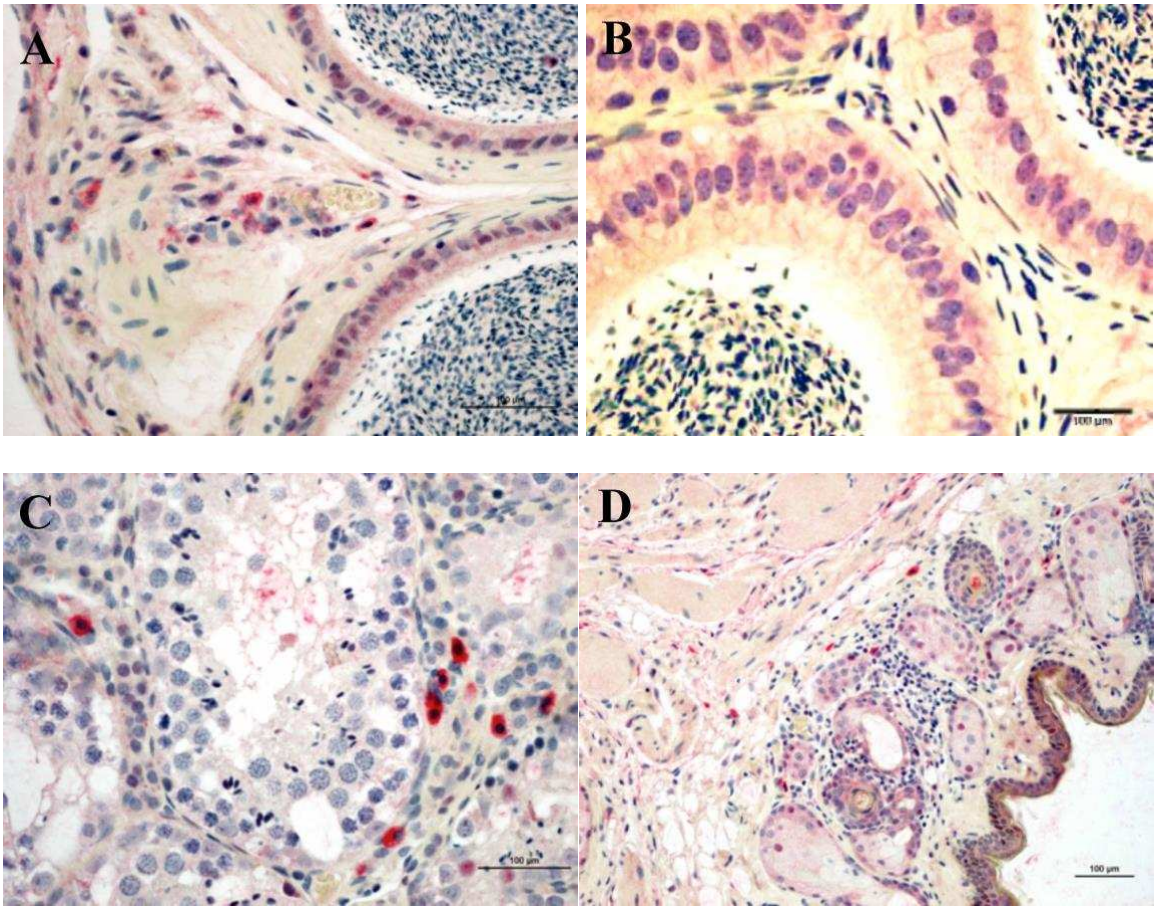

Figure 3.7: Immunoreactivity in AJ-z8, 28 DPI testes, IHC staining for ZIKV antigen compared to negative control bat. A) Tunica albugineal perivascular immunoreactivity mostly in macrophages and fibroblasts of AJ-z8. B) Negative control without immunoreactivity. C) Interstitial immunoreactive mononuclear cells in AJ-z8. D) Focal lymphocytic dermatitis and immunoreactive mononuclear cells in AJ-z8.

3.4: Discussion

Two animal infection experiments were conducted in this investigation; 1) a pilot study to determine susceptibility of Jamaican fruit bats to ZIKV, and 2) a time course study to better understand pathophysiology. The goal was to ascertain whether bats could be used as an animal model for ZIKV pathology and assess the possible role bats might play in viral ecology.

In the pilot experiment, no signs of disease were apparent during the 28 days. Sera collected at euthanasia indicated modest antibody titers of 3200 for each bat by ELISA (table 3.1), whereas the human convalescent control serum titer was 12,800. Bats typically have low to modest antibody titers and this may be because of limited somatic hypermutation and affinity maturation (128, 211-217).

Viruria is commonly detected in ZIKV-infected humans (242) and it may be a more sensitive diagnostic indicator, with higher viral load for a longer period of time compared to blood in both humans and rhesus macaques (239-242). Urine collection from the bats was challenging; however, we were able to collect urine from some of the bats in the time course study. AJ-z6 exhibited viruria only at 3 DPI, and AJ-z7 was equivocal only at 5 DPI, suggesting urine may be a route of viral shedding early in infection.

Blood collection from live bats yields a small volume. In order to assay the serum for viral RNA and perform serology, earlier time points were used to assess for viremia and later time points for seroconversion. Along with sample partitioning for data maximization, the small blood volume generated concern that there would be an undetectably small viral load. To circumvent this issue neat serum and 1:10 diluted serum were inoculated onto Vero cells to amplify virus that may have been present in low numbers. One blind passage on Vero cells was done and cell supernatants assayed by qRT-PCR. The remaining serum for three of the four bats was assayed directly for ZIKV RNA. Cell-serum supernatants, blind passage supernatants, and neat serum results were all negative. Although serum is routinely used to assay for ZIKV diagnostics in humans, it may not be the most sensitive (230, 239-242).

In one investigation ZIKV patient had negative serum for the duration of the study, whereas whole blood yielded positive qRT-PCR results from day nine to day 101 (230). Urine from this patient was positive from the first time point (6 DPI) through 14 DPI and again on day 56. Saliva was positive from day nine through day 14 and again on day 49 (230). Another investigation compared diagnostic samples of 80 positive individuals and showed that urine was positive in 50 of them, whereas serum was positive in only 19 of them by qRT-PCR. The paper concluded that viral loads in urine were ten-fold higher compared to serum and that uremia lasted longer (241). These data corroborated the first study that identified ZIKV shed in urine in which there was a higher viral load in urine for longer duration compared to serum (242). Similar results have been observed in rhesus macaque models. ZIKV RNA in plasma was assayable by qRT-PCR between two and six DPI, but between two to 17 DPI in urine (242). The lack of detectable viremia in the serum of bats may be due to a lack of viremia, diagnostic assays that are not sensitive enough, or the finite blood volume collected preventing proper assay optimization. Additional experiments may be warranted to further investigate the lack of viremia.

ZIKV exhibited tropism for the testes and brain of bats. AJ-z5 at 10 DPI and AJ-z8 at 28 DPI had positive immunoreactivity by immunohistochemistry for ZIKV (figure 3.4, 3.5, & 3.7). Histologically, AJ-z5 testes were within normal limits and AJ-z8 had focal testicular degeneration (figure 3.4) suggesting that viral related pathology may be minimal. AJ-z4 at 5 DPI was negative for ZIKV by IHC and qRT-PCR, but did have focal testicular degeneration on histology, and had IHC positive brain. AJ-z1 at 2 DPI had positive brain tissue by qRT-PCR. Additionally, three of the nine bats (AJ-z6, AJ-z7, AJ-z9) that were negative for ZIKV by IHC and qRT-PCR had histopathology in the hippocampus at later time points (10 and 28 DPI).

While in humans it has yet to be completely elucidated what reproductive organs harbor ZIKV, it has been determined that semen contain ZIKV both in vasectomized and unvasectomized men (230, 233). This suggests that ZIKV is in the testes and/or accessory sex glands. Mouse models have demonstrated

ZIKV infection and associated pathology in the testes (244-246). Limited investigation has been done as to infection of accessory sex glands in mouse models, but one study that assessed the prostate found no virus, possibly due to differential expression of the receptor candidate in the testes but not in the prostate (244). For this experiment, the finding of viral antigen and viral RNA in the testes but not in the prostate is consistent with published animal models.

ZIKV has a predilection for nervous tissue as determined by animal studies and disease manifestation in humans. As a neurological teratogen, ZIKV has been demonstrated in the brain, specifically mononuclear cells, as determined by IHC, of newborns with fatal microcephaly and fetal miscarriages, but no other tissues. Histological lesions included parenchymal calcification, microglial nodules, gliosis, cell degeneration and necrosis (231). In rhesus macaque animal models, viral tropism has been found in peripheral nervous tissue but not the brain itself (247). In immunocompromised mouse models, the virus does have a predilection for the brain but with the mice engineered for specific immune traits it is difficult to know to what extent this emulates natural ZIKV pathophysiology (224). In the bats used in this experiment, evidence of virus and pathology in the brain is consistent with what has been seen in human newborns and fetuses.

In addition to brain and testes on IHC, scrotal skin and mandibular salivary gland harbored viral antigen, suggest that infection of Jamaican fruit bats may recapitulate human infection, which is thought to start with epidermal and dermal cells, and because viral RNA can be detected in human saliva (227, 232). The histopathology for AJ-z5, 5 DPI showed sialadenitis and the presence ZIKV antigen by IHC (figure 3.3). This suggests ZIKV may be shed in the saliva, although additional animal experiments would need to be performed to confirm.

A goal of this study was to determine if Jamaican fruit bats might be a suitable animal model for examining ZIKV infection and pathogenesis. It may be possible to use Jamaican fruit bats to study sexual

transmission, in utero transmission, teratogenesis and neurological pathophysiology. The limitations in using Jamaican fruit bats as an animal model for disease are the slow reproductive rate that may limit the number bats that can be used in a study, and the specific husbandry requirements bats require, which can make them difficult to work with. Furthermore, the immune system of bats appears to be unique in that they constitutively express type I IFNS, and have limited affinity maturation of antibodies, which would lead to differences in the viral-host ecology of bats compared to humans (115, 116, 128, 211-217). Bats harbor many zoonotic pathogens with minimal disease that are BSL-3 and BSL-4 level agents (16, 17, 36, 60, 64, 76). ZIKV, being BSL-2 level virus, may facilitate investigations with greater ease compared to BSL-3 and BSL-4 level viruses of how bats can harbor viruses with minimal disease.

ZIKV is thought to be maintained in two different distinct cycles: sylvatic—cycling between non-human primates (NHP) and mosquito species, and urban—cycling between humans and mosquito species (220). Indeed, it has been demonstrated that numerous NHPs have antibody to ZIKV including numerous monkey species wild trapped near Zika Forest (248), and wild and semi-captive orangutans in Borneo (249). Not only have NHP been found to be seropositive, but also many other mammals, including rodents, horses, cows, and goats (250, 251). Furthermore, experimental inoculation of various North American species resulted in seroconversion (cottontail rabbits, boar goats, pigs, and leopard frogs) and demonstrated viremia (nine-banded armadillo and leopard frogs) (252). Molecular epidemiology suggests animals play an important role in an enzootic cycle (253). Much about the enzootic cycle of ZIKV has yet to be understood but it stands to reason that bats may be capable of maintaining the virus in nature. Jamaican fruit bats are found in northern South America, Central America, and the Caribbean—areas that now have ZIKV potentially exposing bat populations to the virus (223, 237). Wild New World bats, including Jamaican fruit bats, have been found infected with dengue virus and Old World bats have been found with evidence for chikungunya virus infection (185). These viruses, like ZIKV, are predominately vectored by *Aedes aegypti* and *A. albopictus* (186), thus this provides evidence that the vector of ZIKV will take a blood meal from a bat and may infect the animals. However, the data

presented here suggest it is unlikely that bats can serve as reservoir hosts of ZIKV, unless virus sequesters in some as-yet unidentified way that could lead to periodic shedding of virus, at which point ZIKV in bats would be more of a wildlife disease concern with less public health implications. It may also be that some bats become persistently infected and can transmit sexually to maintain virus within populations of bats. Further experimental and field studies will be necessary to fully understand the ecological role of bats in ZIKV maintenance.

CHAPTER 4: SEROLOGICAL EVIDENCE OF ARENAVIRUS CIRCULATION AMONG FRUIT BATS IN TRINIDAD

4.1: Introduction

Tacaribe virus (TCRV) is a New World mammarenavirus first isolated in 1956 from a moribund great fruit-eating bat (*Artibeus literatus*) in Port-of-Spain, Republic of Trinidad and Tobago during a rabies virus surveillance program at the Trinidad Regional Virus Laboratory (TRVL) (154, 254). Eighteen isolates were obtained from six great fruit-eating bats, five purported Jamaican fruit bats (*A. jamaicensis*), and a single isolate from a pool of 344 mixed mosquito species. Of those total nineteen isolates only one of those isolates remains; TRVL-11573 isolated from a great fruit-eating bat. Serological investigation of more than 2,000 mammals found that only bats of the Artibeus genus had evidence of TCRV infection, suggesting that artibeus bats may be reservoir hosts. Work performed about 15 years later also identified seropositive artibeus bats, suggesting TCRV continued to circulate in Trinidad (187). Recent phylogenetic studies suggest that A. jamaicensis bats in Trinidad and Tobago are likely a distinct species, the flat-faced fruit-eating bat (*A. planirostris*) (12). TCRV is closely related to Junin and Machupo viruses (255), the etiologic agents of Argentine and Bolivian hemorrhagic fevers, respectively, and has caused at least one laboratory-acquired human infection (256), raising the possibility that TCRV may have zoonotic potential.

Experimental infections of Jamaican fruit bats resulted in significant neurological disease that manifested as head tremors, inability to roost, remain upright, or fly and mortality. Histopathology revealed mild to moderate neutrophilic interstitial pneumonia, involvement of the spleen and brain in multiple animals. Brain lesions included lymphocytic leptominingitis, mild to moderate multifocal gliosis, or neutrophilic encephalitis. Several inoculated bats were asymptomatic, seroconverted with modest antibody titers by ELISA and low neutralization titers, and appeared to clear virus. These results suggested that infection leads two outcomes; signs of disease that is fatal, or asymptomatic infection

followed by clearance, neither of which is consistent with a typical arenavirus reservoir host (129). In 2014 several new isolates of TCRV were made from lone star ticks (*Amblyomma americanum*) in central Florida (188) where artibeus bats are not found. These isolates were genetically distinct from TRVL-11573 and include an additional 12 amino acids and several other polymorphisms in the glycoprotein (257, 258). However, it is likely that TRVL-11573 accumulated mutations during its 20 passages in suckling mice (figure 4.1) and subsequent passage in Vero cells for preparation of viral stocks. Together, these data argue against artibeus bats as reservoirs of TCRV, and suggest the TCRV is likely hosted by another species, perhaps multiple species, and that spillover to bats results in disease.

We were interested in determining if artibeus bats are reservoir hosts of TCRV, to determine if the virus continues to circulate among bats in Trinidad and, if so, whether new isolates of the virus could be obtained. We collected blood and tissue samples from bats and performed serology, PCR and attempted virus isolation from tissues. Although many bats had antibodies reactive to recombinant TCRV nucleocapsid, none of the tested samples had neutralizing antibodies or viral RNA, and no isolates of TCRV were obtained. These findings are congruent with experimental infection of Jamaican fruit bats and further indicates that bats are not natural reservoir hosts of Tacaribe virus.

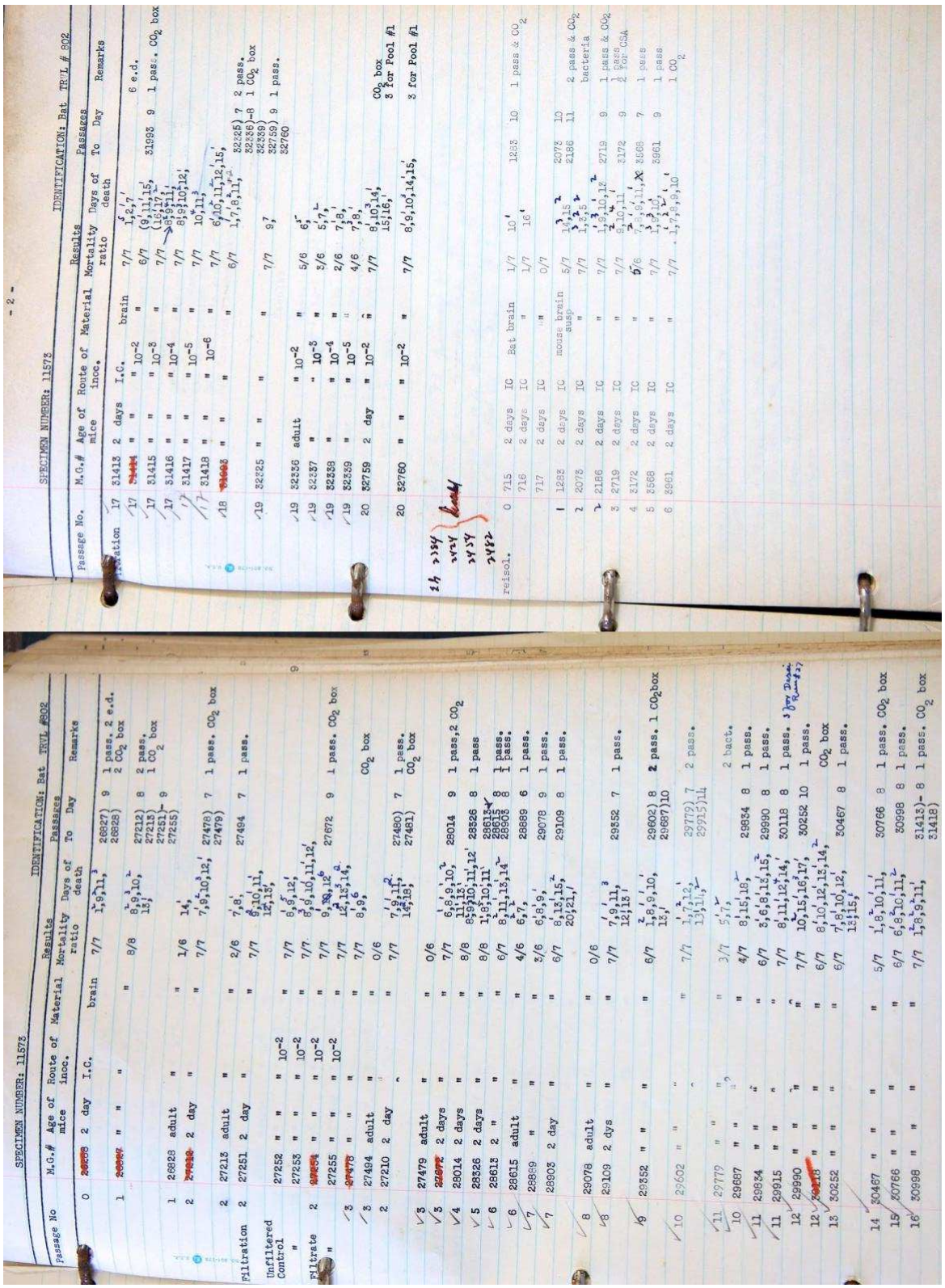

Figure 4.1: Passage history of Tacaribe virus isolate TRVL-11573. Obtained from the library archive of the Caribbean Epidemiology Centre (CAREC), Port of Spain, Trinidad and Tobago. TCRV-11573 was isolated from bat TRVL #802 (great fruit-eating bat, A. literatus) and intracranially passaged in newborn mice (unknown strain). Twenty passages were made to generate the first stock of TCRV-11573.

4.2: Methods and Materials:

4.2.1: Bats

Bats were captured with mist nets in February, 2012 for sampling, with approval of the Ethics Committee, Faculty of Medical Sciences, University of the West Indies, St. Augustine Campus, and under a special game license from the Wildlife Section, Forestry Division, Ministry of Agriculture, Land and Fisheries, Republic of Trinidad and Tobago. Trap sites were Mount Hope (N 10.67120, W 061.28677), Lopinot (N 10.69792, W 061.32243), Santa Cruz (N 10.69596, W 061.44629), and Maracas Valley (N10.70945, W061.40177) (figure 4.2). No threatened or endangered species were captured. Live bats were individually placed in cloth bags for transport to laboratory facilities at the University of the West Indies, St. Augustine for processing.

Figure 4.2: Locations of bat collections. Mt. Hope bats were collected during the day from a building roost on the campus of The University of West Indies. Collections from the remaining three sites were performed on three consecutive nights.

Bats were humanely euthanized by inhalation of isoflurane and thoracotomy prior to tissue harvesting at necropsy. Cardiac blood was collected when the heart was visualized upon thoracotomy and transferred to serum separator tubes (SST). SSTs were inverted ten times, sat at room temperature for one hour and then centrifuged at 1000 x g for 10 minutes at room temperature. Serum was removed from the clot, placed in a sterile microcentrifuge tube and stored at -20°C. All personnel were immunized against rabies virus and appropriate PPE was worn during collections, and bat necropsies were performed in a class II biosafety cabinet. Samples were immediately frozen and stored at -80°C prior to shipment on dry ice to Colorado State University (Fort Collins, CO, USA) and Rocky Mountain Laboratories (Hamilton, MT, USA) for further processing. Cytochrome B PCR sequencing was performed on DNA from the artibeus bats to identify species.

4.2.2: Virus Isolation

Lung tissue was processed for attempted virus isolation of TCRV. Tissues measuring approximately 2 mm^3 were individually placed into 1.5-ml screw cap tubes with 500 ul 5%FBS-DMEM and a 5 mm stainless steel ball, and homogenized on TissueLyser LT (Qiagen, Valencia, CA) for 5 minutes at 2 Hz to minimize heat and potential virus inactivation. Tissues were placed on ice and then homogenized again to ensure complete tissue disruption. Tissues were centrifuged at 3000 rpm for 5 min at 4°C. Four hundred microliters of supernatant was added to 500 ul 5%FBS-DMEM and filtered through 0.2 um Acrodisc filter. One hundred microliters of filtrate was inoculated onto confluent Vero E6 cells in 24 well plates. Inoculated cells were incubated for one hour at 37°C and then 1 ml of 2% FBS-DMEM added to each well. Cells were maintained in an incubator at 37°C with 5% CO_2. Fresh medium was added on day six. After ten days of incubation, RNA was extracted from supernatants with the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instructions. The homogenized tissue pellets were used for RNA extractions by adding 350 ul RLT with 1% 2ME and homogenized 2 x 5 min at 50 Hz. Tissue was centrifuged at 3000 rpm for 5 min at 4°C. Supernatant was processed for RNA extraction with the RNeasy Mini kit with Qiashredder (Qiagen, Valencia, CA) according to

manufacturer's instructions. Control TCRV (TRVL-11573) RNA was isolated from inoculated Vero E6 stocks. RNA samples were kept at -80 °C until further use.

4.2.3: Reverse Transcription and conventional PCR

RNA was reverse transcribed with random primers using Quantitect Reverse Transcription kit (Qiagen, Valencia, CA) according to manufacturer's instructions. PCR was performed using TCRV primers (forward, 5'-TGTGGCTTTCTGAAGCAGTG-3'; reverse, 5'-AGGCTCTCGATCGCAAATTA) and PCR Core Kit (Qiagen, Valencia, CA) as previously described (129). Amplification conditions were melting for 3 min at 94°C, 35 cycles at 94°C for 30 s, and annealing at 54 °C for 30 s, and extension at 72°C for 1 min. Reactions were held at 72°C for 10 minutes. Samples were resolved on 1% agarose gels.

4.2.4: ELISA and Western Blot

Recombinant His-tagged TCRV nucleocapsid antigen (259) was expressed in E. coli BL21(DE3) (260) and used for ELISA and western blot. ELISA was performed as previously described using a protein-A/G-HRP conjugate (260). Sera were diluted 1:100 in PBS for the ELISA. Samples with an absorbance of 3 times greater than the background absorbance were considered positive. Endpoint titers of positive samples were determined by ELISA, and antibody reactivity validated (1:500) by western blot, also detected with protein-A/G-HRP (Thermo Fisher Scientific, Waltham, MA). Control sera were from archived samples collected from a previous experimental infection of Jamaican fruit bats (129).

For the western blot, 20 ug of TCRV nucleoprotein was combined with 4X NuPAGE LDS sample buffer (Thermo Fisher Scientific, Waltham, MA), and deionized sterile water to denature protein and reduce protein disulfide bonds. The mixture was heated at 72°C for 10 minutes. A NuPAGE 4-12% BT 1.0 mm (single well) gel (Thermo Fisher Scientific, Waltham, MA) was loaded with TCRV nucleoprotein and ran for 60 minutes at 200 volts with 1X SDS running buffer and NuPAGE antioxidant (Thermo Fisher Scientific, Waltham, MA). The gel was removed from the gel tank and a membrane was

dampened in methanol and placed on the gel. TCRV nucleoprotein was allowed to transfer for five hours at 150 volts. The membrane with the TCRV nucleoprotein was then removed and cut into strips. A 1:500 dilution was made for each bat serum sample in 5% BSA-TBS. Antibody to the His-tag was used as a control. A membrane strip was incubated in individual bat serum samples and one strip in His-tag antibody for 24 hours at 4°C. Strips were washed 3x in TBS + Tween buffer. A 1:5000 dilution of protein AG in 5% BSA-TBS was prepared and strips incubated in protein AG dilution for 2 hours. Strips were washed 3x in TBS + Tween buffer. TMB membrane peroxidase system was prepared and dropped onto strips. Strips were incubated for 15 minutes and then washed 3x with TBS + Tween buffer (KPL, Gaithersburg, MD) and bands visualized.

4.2.5: Serum Neutralization

TCRV TRVL-11573 was used for serum neutralization testing. Sera were diluted 1:10 in 2% FBS-DMEM in the first well and a $\log_2$ dilution series was prepared for each sample. TCRV (10^2 $TCID_{50}$) was added to each well in 100 ul (1:20 final dilution of serum) for 1 hour at 37°C, then the entire volume (200 ul) transferred to 96 well plates containing confluent Vero cells. Fresh media was added on day seven. Plates were scored for cytopathic effect on day seven.

4.3: Results

4.3.1: Bats

Cytochrome B sequencing showed that artibeus bats were *A. planirostris* or *A. literatus*; no *A. jamaicensis* bats were collected (data not shown). Of the six species captured, four had seropositive animals; the flat-faced fruit bat (*A. planirostris*), great fruit-eating bat (*A. literatus*), Seba's fruit bat (*Carollia perspicillata*), and yellow-shouldered fruit bat (*Sturnira lilium*) (table 4.1).

Table 4.1: Distribution and serology of study bats of Trinidad.

Animal ID	Site[1]	Species	Sex (M/F/I)	Adult/Juvenile	ELISA TT[2]	WB[3]
1	Mt. Hope	*A. planirostris trinitatis*	F	A	-	nd
2	Mt. Hope	*A. planirostris trinitatis*	U	J	-	nd
3	Mt. Hope	*A. planirostris trinitatis*	F	A	100	+
4	Mt. Hope	*A. planirostris trinitatis*	U	J	100	+
5	Mt. Hope	*A. planirostris trinitatis*	F	A	-	nd
6	Mt. Hope	*A. planirostris trinitatis*	F	A	-	nd
7	Mt. Hope	*A. planirostris trinitatis*	U	J	200	+
8	Mt. Hope	*A. planirostris trinitatis*	F	A	-	nd
9	Mt. Hope	*A. planirostris trinitatis*	F	A	200	+
10	Mt. Hope	*A. planirostris trinitatis*	F	A	100	+
11	Mt. Hope	*A. planirostris trinitatis*	F	A	-	nd
12	Mt. Hope	*A. planirostris trinitatis*	M	J	-	nd
13	Mt. Hope	*A. planirostris trinitatis*	M	J	-	nd
14	Mt. Hope	*A. planirostris trinitatis*	F	A	-	nd
15	Mt. Hope	*A. planirostris trinitatis*	M	J	-	nd
16	Mt. Hope	*A. planirostris trinitatis*	M	J	-	nd
17	Mt. Hope	*A. planirostris trinitatis*	M	A	100	+
18	Mt. Hope	*A. planirostris trinitatis*	F	A	200	+
19	Mt. Hope	*A. planirostris trinitatis*	M	J	-	nd
20	Mt. Hope	*A. planirostris trinitatis*	F	A	-	nd
21	Mt. Hope	*A. planirostris trinitatis*	M	J	-	nd
22	Mt. Hope	*A. planirostris trinitatis*	M	J	-	nd
23	Mt. Hope	*A. planirostris trinitatis*	F	J	-	nd
24	Mt. Hope	*A. planirostris trinitatis*	F	A	-	nd
25	Mt. Hope	*A. planirostris trinitatis*	F	J	-	nd
26	Mt. Hope	*A. planirostris trinitatis*	M	J	-	nd
27	Mt. Hope	*A. planirostris trinitatis*	M	J	-	nd
28	Lopinot	*A. planirostris trinitatis*	F	A	-	nd
29	Lopinot	*A. planirostris trinitatis*	M	J	100	+
30	Lopinot	*A. literatus*	F	A	200	+
31	Lopinot	*A. literatus*	M	J	-	nd
32	Lopinot	*A. literatus*	M	J	-	nd
33	Lopinot	*A. literatus*	F	J	-	nd
34	Lopinot	*A. literatus*	F	A	-	nd
35	Lopinot	*A. literatus*	F	A	-	nd
36	Lopinot	*A. literatus*	F	J	-	nd
37	Lopinot	*A. literatus*	F	A	400	+
38	Lopinot	*A. literatus*	M	J	400	+
39	Lopinot	*A. literatus*	F	J	-	nd
40	Lopinot	*A. literatus*	F	A	-	nd
41	Lopinot	*A. literatus*	M	A	800	+
42	Lopinot	*Glossophaga soricina*	F	A	-	nd
43	Lopinot	*Glossophaga soricina*	F	A	-	nd
44	Lopinot	*A. planirostris trinitatis*	M	A	200	+
45	Lopinot	*A. literatus*	M	A	-	nd
46	Lopinot	*A. literatus*	M	A	-	nd

47	Lopinot	*Sarcopteryx bilineata*	M	A	-	nd
48	Lopinot	*Glossophaga soricina*	F	A	-	nd
49	Lopinot	*Sturnira lilium*	M	A	200	+
50	Lopinot	*Sturnira lilium*	F	A	100	+
51	Lopinot	*Sturnira lilium*	F	A	-	nd
52	Lopinot	*Sarcopteryx bilineata*	M	J	-	nd
53	Lopinot	*A. literatus*	M	A	200	-
54	Lopinot	*A. planirostris trinitatis*	M	A	-	nd
55	Santa Cruz	*A. literatus*	M	A	-	nd
56	Santa Cruz	*A. literatus*	M	A	200	-
57	Santa Cruz	*Sarcopteryx bilineata*	M	A	-	-
58	Santa Cruz	*Sarcopteryx bilineata*	F	A	-	-
59	Santa Cruz	*A. literatus*	M	A	200	+
60	Santa Cruz	*A. literatus*	M	A	-	-
61	Santa Cruz	*A. literatus*	M	A	200	
62	Santa Cruz	*A. literatus*	M	A	-	nd
63	Santa Cruz	*A. planirostris trinitatis*	M	A	400	+
64	Santa Cruz	*A. literatus*	M	A	100	+
65	Santa Cruz	*A. literatus*	M	A	100	+
66	Santa Cruz	*A. planirostris trinitatis*	M	A	-	nd
67	Santa Cruz	*Sarcopteryx bilineata*	F	A	-	nd
68	Santa Cruz	*Sarcopteryx bilineata*	F	A	-	nd
69	Santa Cruz	*A. literatus*	M	A	200	-
70	Santa Cruz	*A. planirostris trinitatis*	M	A	-	+
71	Santa Cruz	*A. literatus*	M	A	-	+
72	Santa Cruz	*A. planirostris trinitatis*	M	A	400	+
73	Santa Cruz	*Sarcopteryx bilineata*	M	A	200	-
74	Santa Cruz	*A. literatus*	M	A	-	+
75	Santa Cruz	*A. literatus*	M	A	200	+
76	Santa Cruz	*A. literatus*	M	A	-	+
77	Maracas Valley	*C. perspicillata*	M	A	100	+
78	Maracas Valley	*C. perspicillata*	F	A	200	+
79	Maracas Valley	*A. planirostris trinitatis*	F	A	400	nd
80	Maracas Valley	*A. literatus*	M	A	-	nd
81	Maracas Valley	*A. literatus*	M	A	-	nd
82	Maracas Valley	*C. perspicillata*	M	A	-	-
83	Maracas Valley	*A. literatus*	F	A	100	-
84	Maracas Valley	*C. perspicillata*	F	A	400	-
Control	673 45 DPI	*A. jamaicensis*	pos ctrl		1600	+
Control	678 45 DPI	*A. jamaicensis*	pos ctrl		800	+
Control	679 45 DPI	*A. jamaicensis*	pos ctrl		1600	nd
Control	680 2 DPI	*A. jamaicensis*	neg ctrl		-	-
Control	681 2 DPI	*A. jamaicensis*	neg ctrl		-	nd

[1]Locations of collections are shown on Figure 1.
[2]Titer represents the reciprocal of the greatest dilution with signal.
[3]+, positive; -, negative; +/-, equivocal; nd, not done. Insufficient sample remained to test bat 61 by western blot.
[4]Dark grey boxes are ELISA-positive adults, whereas light grey boxes are ELISA-positive juveniles.

4.3.2: Virus Isolation and PCR

Lung tissue was assayed for TCRV RNA via PCR because results from experimental infections of artibeus bats with TCRV demonstrated that, while many organ types yield virus after infection, lung tissue was the most consistent location of viral infection. All PCR results were negative for TCRV.

The pellets of lung homogenates and supernatants of inoculated Vero cells were also screened for TCRV RNA and none of the samples had amplicons (data not shown).

4.3.3: Serology

Of the six species captured, four had seropositive animals; the flat-faced fruit bat, great fruit-eating bat, Seba's fruit bat, and little yellow-shouldered bat (table 4.2). Although Seba's fruit bats and little yellow-shouldered bats higher seroprevalence rates than either artibeus species, the sample sizes for each was low.

Table 4.2: Seroprevalence to TCRV among adult bats of Trinidad.

Species	Diet	Ab positive (Adults)	Sampled (Adults)	Prevalence (%)
Artibeus literatus	frugivore	8	25	32
A. planirostris trinitatis	frugivore	8	21	38
Carollia perspicillata	frugivore	2	4	50
Sarcopteryx bilineata	insectivore	0	6	0
Glossophaga soricina	nectarivore	0	3	0
Sturnira lilium	frugivore	2	3	67

Twenty-seven of the 62 adult bats were seropositive by initial ELISA screening and titers ranged from 100 to 800 (table 4.3). However, western blot results indicated that only 22 of the bats had antibody

specific (figure 4.3) to TCRV nucleocapsid antigen, and two artibeus bats (accession numbers 70 and 74) were ELISA-negative but WB-positive. Insufficient serum was available to perform WB analysis on bat 61 that was ELISA-positive. Antibodies were detected in some juveniles and may represent maternal antibody. Control sera from bats used in a previous experimental infection (129) had titers between 800 and 1600.

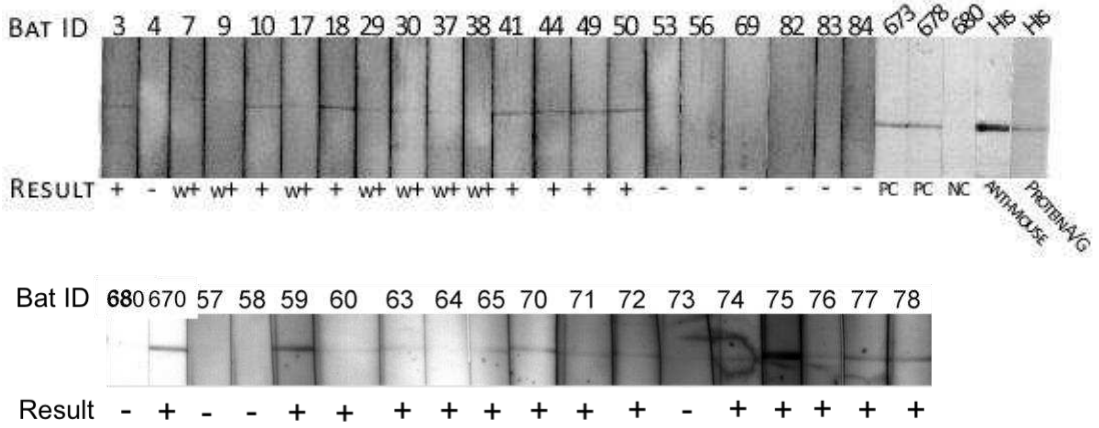

Figure 4.3: Western blot results on a subset of samples. Bat identifications are listed on the top of each western blot and results on the bottom. "His" represent controls targeting the his-tag. PC indicates a positive control bat. NC indicates a negative control bat.

Serum neutralization was used to assess if these bats had neutralizing antibody to the glycoprotein. No bats yielded a neutralizing antibody to TCRV.

Screening ELISAs were performed on all bat samples. Thirty three out of the 89 samples had ODs greater than the negative cut off of 0.300. Those that were positive were titered for further exploration by ELISA. Titers on positive animals ranged from 100 to 800. A subset of samples (bat id: 680—negative control, 678—positive control, 673—positive control, 57, 58, 59, 60, 63, 64, 65, 70, 71, 72, 73, 74, 75, 76, 77, 78, 3, 4, 7, 9, 10, 17, 18, 29, 30, 37, 38, 41, 44, 49, 50 53, 56, 69, 82, 83, 84) were assayed with a western blot for validation. Eleven were negative, 27 were positive. Results were consistent with the ELISA. These assays used the nucleocapsid protein.

4.4: Discussion

Generally speaking, suitable vertebrate reservoirs of viruses, which have been best studied in rodents (261-265), have little to no pathology and remain persistently infected for extended periods of time, perhaps for life with some viruses and their hosts. Although few studies of bats as viral reservoirs have been performed, those that have typically demonstrate this pattern (37, 266, 267). Perhaps the best studied virus/reservoir host systems are the rodent-borne hantaviruses, which are similar to arenaviruses, in which the viruses establish apathogenic infections without eliciting aggressive immune responses (261, 268, 269). In each of these systems, the viruses do not cause meaningful pathology and persist for many months or longer, and heterologous hantavirus inoculation of a reservoir host species also result in innocuous infections (270, 271).

Historically, the natural reservoir host(s) of TCRV has been presumed to be artibeus bats (154, 187). All other mammarenaviruses with known reservoirs are hosted by rodents (272), thus the hypothesis that bats may serve as a reservoir of TCRV is unusual. We have obtained serological evidence that TCRV or similar arenavirus is circulating in at least two, and possibly four species of fruit bats in Trinidad; flat-faced fruit bats, great fruit-eating bats, Seba's fruit bats and yellow-shouldered fruit bats. Flat-faced fruit bats, great fruit-eating bats and yellow shouldered fruit bats have previously been identified as having antibodies to TCRV (154, 187) but identification of antibodies in Seba's fruit bat has not been reported until now. We did not capture any Jamaican fruit bats (*A. jamaicensis*), which is one of the two species originally identified as a host when TCRV isolates were first made in the 1950s (154). Considering other evidence that Jamaican fruit bats are not found in Trinidad (12), it is likely that flat-faced fruit bats were misidentified as Jamaican fruit bats in the original paper.

Viral RNA was not detected in the lung tissues of bats or in Vero cells inoculated with clarified lung homogenates, nor was virus isolated from blind passage on Vero cells, suggesting that bats do not serve as reservoir hosts for TCRV. We previously performed experimental infections of Jamaican fruit

bats with TCRV TRVL-11573 and determined that high doses of virus caused disease with high fatality rates but that low dose virus resulted in clearance without conspicuous disease (129). Interestingly, surviving bats in this study had only modest antibody titers by ELISA (table 4.1) and very low neutralizing titers (129). Little brown bats (*Myotis lucifigus*) do not appear to substantially use somatic hypermutation (SHM), suggesting that affinity maturation (which accounts for high antibody titers) may be limited (123). If SHM is limited in other bat species, it could account for the low titers we observed in this study.

Considering the serological evidence presented here that at least four species of fruit bats and a previous study showing other bat species (187) are susceptible to arenaviruses, it may be that bats are spillover hosts from rodent reservoirs. The high seroprevalence suggests that after spillover the virus may be transmitted among bats and considering the high densities and direct contact among individuals within a colony, transmission could occur. However, experimental infections showed that despite detectable viral RNA in oral and rectal swabs for several weeks, transmission to sentinel bats did not occur (129). It may be that the artificial housing in our experimental model disrupted the natural biology of the bats (e.g., confined to cages, behavioral changes, dietary differences, etc.) that may have prevented transmission.

More than 60 species of bats are found in Trinidad; thus, we cannot exclude the possibility that another bat species in Trinidad may be a reservoir host of TCRV. Trinidad is 12 km from Venezuela and it is possible that TCRV may move between Trinidad and Venezuela (273). The next closest nation to Trinidad and Tobago is Grenada, where artibeus bats are also found. However, the flight distance between the islands is about 160 km, making it difficult for artibeus bats to routinely migrate between the islands because their range is about 15 km (273).

The recent isolation of TCRV from lone star ticks in central Florida also suggests that artibeus bats are not reservoir hosts. Lone star ticks are not known to feed on bats; however, they routinely feed on

rodents and other terrestrial mammals (274). Trinidad and Florida do not share common species of rodents or bats, other than introduced house mouse (*Mus musculus*) and Norway rats (*Rattus norvegicus*), neither of which have been shown to host TCRV. Several Cricetidae rodent species are found in Trinidad and Florida, and many arenaviruses are hosted by members of this family. Insectivorous velvety free-tailed bats (*Molossus molossus*) are common in Trinidad but found only in the Florida Keys, suggesting it is not the source of TCRV isolated from central Florida. TCRV may have multiple natural reservoir host species, which is unusual but not unprecedented among the mammarenaviruses (272). The original paper describing TCRV's isolation did not identify rodents as potential reservoirs; however, it is unclear as to how many species and individuals were sampled (154). Thus, the accumulated evidence suggests that artibeus bats are not reservoir hosts of Tacaribe virus.

CHAPTER 5: *DE NOVO* ASSEMBLY OF THE JAMAICAN FRUIT BAT (*ARTIBEUS JAMAICENSIS)* GENOME

5.1: Introduction

Several bat genomes are currently available but fail to include Jamaican fruit bats (*Artibeus jamaicensis*) (275). The transcriptome for Jamaican fruit bats has been sequenced and annotated (276). In order to progress the laboratory tools available to research Jamaican fruit bats and compliment the transcriptome *de novo* assembly was conducted. An Illumina platform was used to draft the genome sequence with short read sequences. *De novo* assembly was performed using Soapdenovo2.

5.2: Methods and Materials

5.2.1: Genomic DNA Extraction

Genomic DNA was extracted from 8th passage Jamaican fruit bat primary kidney epithelial cells. The cell line was developed from a mature, male Jamaican fruit bat. The *Quick*-DNA™ Miniprep Plus Kit (Zymo Research, Irvine, CA) was used according to manufacturer's protocol to extract genomic DNA.

5.2.2: Genomic DNA Library Preparation and Sequencing

Genomic DNA was sheared through sonification using a Covaris instrument (Covaris, Inc., Woburn, MA) with settings appropriate for target fragments of 500 bp. KAPA Hyper Prep Kit (Kapa Biosystems, Inc., Wilmington, MA) was used to prepare the library according to protocol instructions. Product was amplified using two cycles of PCR. Product was resolved on a 1% gel and excising product between 300 and 600 bp. DNA was recovered with Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA). Fragment size and gDNA quality was confirmed with Agilent 4200 TapeStation (Agilent, Santa Clara, CA). Illumina NextSeq 500 (Illumina, Inc., San Diego, CA) sequencing platform was used for short read sequencing.

5.2.3: *De Novo* Assembly

FastQC Version 0.11.5 was used to assess quality of the sequences both before and after trimming (277). Files were trimmed using Trimmomatic-0.33 with the following settings: ILLUMINACLIP: NexteraPE-PE.fa:1:30:10:4: true LEADING:20 TRAILING:20 SLIDINGWINDOW:4:25 MINLEN:60 (278). Soapdenovo2 was then used to perform assembly with the following parameters: max_rd_len=150,[LIB], avg_ins=580, reverse_seq=0, asm_flags=1, rd_len_cutoff=150, rank=1, pair_num_cutoff=3, map_len=32 (279). A genome quality report was generated with QUAST 4.6.0 (280).

5.3: Results

5.3.1: Quality Assessment Pre- and Post-Trimming

FastQC Version 0.11.5 was used to assess quality of raw sequence data both before and after trimming to both target any sequencing errors that may have arisen during library preparation, and sequencing, and to determine effectiveness of trimming. Prior to trimming the data had acceptable scores by all parameters measured by FastQC; however, trimming improved the quality of the raw data (figure 5.3.1).

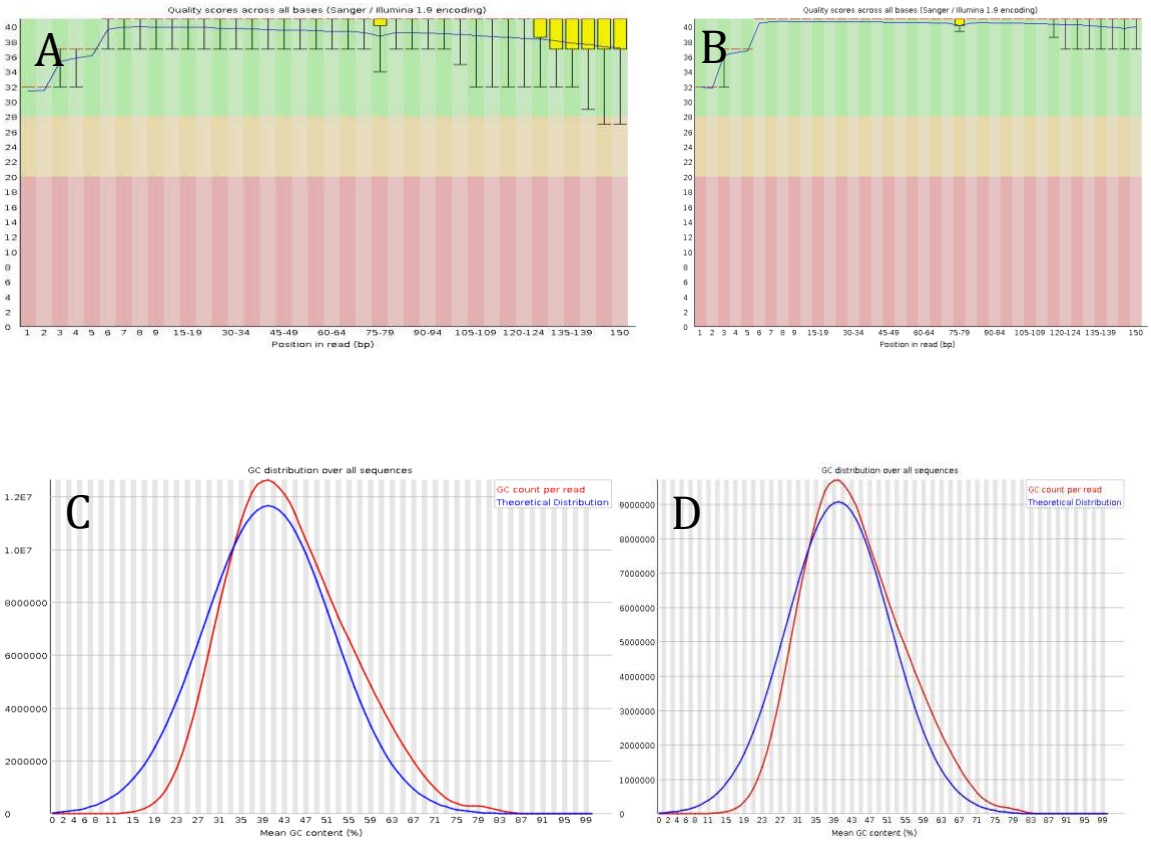

Figure 5.1: Quality of reads pre- and post-trim. (A) Pre-trim run one compared to (B) Post-trim run one shows an increase in quality score (y-axis) across all bases (x-axis). (C) Pre-trim run one compared to (D) Post-trim run one demonstrates the normal GC distribution both before and after trimming indicating quality of sequence is high both before and after trimming.

5.3.2: *De Novo* Assembly

Soapdenovo2 was used to assemble the genome of the Jamaican fruit bat. QUAST 4.6.0 was used to generate a quality report (figure 5.2) (280).

	AJgenome_assembly
# contigs (>= 0 bp)	6455864
# contigs (>= 1000 bp)	480065
# contigs (>= 5000 bp)	15361
# contigs (>= 10000 bp)	790
# contigs (>= 25000 bp)	0
# contigs (>= 50000 bp)	0
Total length (>= 0 bp)	2335995929
Total length (>= 1000 bp)	942870564
Total length (>= 5000 bp)	101925200
Total length (>= 10000 bp)	9383853
Total length (>= 25000 bp)	0
Total length (>= 50000 bp)	0
# contigs	1173816
Largest contig	22536
Total length	1425433322
GC (%)	40.06
N50	1396
N75	837
L50	283990
L75	618234
# N's per 100 kbp	0.00

Figure 5.2: Quality report of Jamaican fruit bat genome generated by QUAST 4.6.0.

5.4: Discussion

De novo sequencing of the Jamaican fruit bat genome generated a 1.4 gigabase (Gb) genome with 20x coverage. Comparatively, published bat genomes of different species are ~2.0 Gb (275). Bat genomes are much smaller than other mammalian genomes, and more similarly sized to bird genomes; possibly a prelude to flight (281). A quick gene analysis indicated that some expected genes were missing or incomplete. Given the coverage and genome size, the genome is incomplete. In the future, long-read sequencing may be performed on a new library, and assembled with this short-read sequence in order to improve the over-all quality of this genome. Given the intention of *de novo* sequencing of the Jamaican fruit bat genome was to identify sequences to advance laboratory capabilities, such as primer design and cloning, this sequence provides a platform with which to do this.

CHAPTER 6: CONCLUDING REMARKS

An overwhelming amount of evidence exists that identify bats as having a unique, commensal relationships with numerous viruses, particularly viruses with vast public health implications such as Ebola virus, SARS-CoV, and Nipah virus, amongst others. The principal goal of this dissertation research was to better understand New World bats and their relationship with novel viruses to add to the repertoire of data that investigates bats as reservoir species.

The present study is the first to conduct *in vivo* experiments with novel influenza A viruses (IAV) H17N10 and H18N11 in bats. H17N10 was first discovered in little yellow-shouldered bats belonging to the genus *Sturnira*. Inoculation of Jamaican fruit bats with rescued H17N10 did not result in evidence to support the hypothesis that Jamaican fruit bats were susceptible to the virus. It was postulated that the evolutionary divergence between little yellow-shouldered bats was great enough, and the virus specific enough, so that Jamaican fruit bats could not be infected. A time-course study was conducted with H18N11 in Jamaican fruit bats. Jamaican fruit bats inoculated with H18N11 shed virus rectally but not orally, viral RNA was detected in the gastrointestinal tract, and histology revealed cellular infiltrates in the small intestine of bats during time points prior to 17 DPI. At and after 17 DPI bats seroconverted, detected by ELISA. Furthermore, two uninoculated bats were exposed to inoculated bats to investigate transmission potential. Rectal swabs collected from these naïve bats also detected viral RNA by qRT-PCR at similar time points early in course of infection. Both inoculated bats and uninoculated bats seroconverted. These data delineate pathophysiology that is reminiscent of IAV in their reservoir hosts, waterfowl, and low pathogenic avian influenza virus in gallinaceous birds. Transmission of H18N11 in bats most likely occurs fecal-orally, virus has tropism for tissues of the gastrointestinal tract, bats mount an adaptive immune response and virus is cleared. It has been suggested that bats may serve as an additional reservoir for IAVs, but given that no human has been diagnosed with H18N11 infection and

the high level of bat influenzas from canonical IAVs, this is unlikely. More aptly H18N11 is an endogenous virus to bats and circulates amongst bat populations causing minimal disease.

This study is furthermore the first to explore New World bat as a reservoir for Zika virus (ZIKV). Given the temporal overlap of ZIKV emerging endemicity in the Western Hemisphere with the native range of Jamaican fruit bats, the fact that bats have evidence for infection with dengue and chikungunya virus, the *Aedes* vector for ZIKV will feed on bats, and that an enzootic wildlife reservoir for ZIKV is suspected but not identified, it is plausible that bats may be susceptible to infection and serve as a reservoir. Additionally, the experiment was conducted to investigate the potential of bats to function as an animal model. A time-course study was conducted to observe patterns in pathophysiology. Bats seroconverted, albeit low titers, and viral tropism was demonstrated for testes and brain tissues. Furthermore, viral RNA was detected in urine, and a salivary gland was IHC positive, offering two routes of shedding in bats. ZIKV is predominately vectored through mosquitoes but has also been transmitted vertically and horizontally in humans. Bats are gregarious, roost in great numbers on top of each other; they are rather untidy animals, that drop partially eaten fruit, and urinate frequently on other bats. While documentation of exposure to virus positive urine and saliva does not exist, due to the natural behavior of bats, they would be the ideal species to facilitate such transmission. Evidence for ZIKV was not detected in the serum. This may be because bats do not become viremic, serum may not be the most sensitive diagnostic sample (as demonstrated in some human studies and animal-model studies), and/or the small size of bats produced a finite blood volume and there were not enough sera to fully optimize viral detection. The lack of evidence for viremia, if true, would prevent mosquitoes from serving as a vector for ZIKV and indicates that bats may not be a viable reservoir for human infection. ZIKV may still circulate amongst bats through non-mosquito-vectored transmission routes, making it a wildlife disease problem. While tissue tropism in bats recapitulates human infection, immunological research demonstrates that bats constitutively express type I IFNs, and may not have affinity maturation. This

would make they respond to viral infection different from humans, and infers that bats may not be the best animal-model for human disease.

Both models of viral infection, H18N11 and ZIKV in Jamaican fruit bats, may be excellent systems to better understand the relationship between bats and their viruses given logistical accessibility as BSL2 viruses, and the ability to draw upon one of the few experimental captive bat colonies in the world. Furthermore, both studies detail suspected incidental pathology in Jamaican fruit bats. As more research is conducted in bats it is important to know what pathological abnormalities bats are prone to in order to separate out background lesions from lesions of interest, therefore it is necessary to describe all pathology. These studies identified frequent lesions in cardiac muscle and lung congestion. The high-energy expenditure of flight, and a cardiovascular system that must function at a level to accommodate these energy expenditures, may predispose bats to heart-related issues. Lung congestion was observed in negative control bats that were kept in the same style of cage that inoculated bats were housed in. Lung congestion has not been observed in bats euthanized, necropsied and examined histologically directly from the free-flight colony room. Restricting normal mobility and flight may predispose bats to lung disease. Another difference between free-flight colony bats, and caged inoculated bats were the rooms they were kept in. The colony room is often warmer and more humid than the inoculation room. Given that bats are heterotherms, the difference in ambient temperatures may play a role in lung pathology. The mechanism for this will be important to ascertain for future studies as to prevent pathology that might interfere with viral-host ecology.

This study also provides novel evidence that TCRV, or a similar virus, may still be circulating in Trinidad. Furthermore, the research presented here expands the host range of TCRV in bats as previously flat-faced fruit bats, great fruit-eating bats, yellow shouldered-fruit bats have been found seropositive for a TCRV-like virus, but not Seba's fruit bat—which we found to have antibodies to TCRV.

These studies explore novel viruses in New World bats and further our understanding of the relationship of bats with their viruses. This is paramount given the capacity of bats to serve as reservoirs for emerging infectious diseases and the anthropogenic rate of biodiversity loss and human encroachment on wildlife species thereby increasing potential exposure to novel pathogens. The more scientists understand about bats, public health, and disease ecology the more it is possible to intervene to both mitigate spillover and protect bat species all over the world.

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