DISSERTATION

CHARACTERIZING THE EFFECTS OF BLUETONGUE VIRUS COINFECTION IN *CULICOIDES SONORENSIS*

Submitted by

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ABSTRACT

CHARACTERIZING THE EFFECTS OF BLUETONGUE VIRUS COINFECTION IN *CULICOIDES SONORENSIS*

Bluetongue virus (BTV) is a segmented, double-stranded RNA virus transmitted by *Culicoides* biting midges. Infection of domestic and wild ruminants with BTV can result in devastating disease and significant economic losses. In concert with climate change, BTV outbreaks have been characterized by an expanding geographical range and incursions of novel serotypes into endemic regions. As a virus with a segmented genome, reassortment between BTV strains may increase genetic diversity which can alter BTV transmission dynamics and generate epizootic events. While factors driving BTV's expansion are poorly understood, reassortment between virus strains may enhance BTV's ability to spread to new regions. The following studies aimed to investigate different facets of BTV coinfection and reassortment in the *Culicoides* vector including temperature effects, BTV serotype infection titers, and virus coinfection dissemination.

While warmer temperatures have been demonstrated to increase virogenesis, temperature effects on reassortment is not known. The first aim was to evaluate how temperature affects *Culicoides* survivorship, virogenesis, and progeny virus genotype outcomes in BTV coinfected *Culicoides sonorensis*. *C. sonorensis* were provided bloodmeals containing BTV serotype 10 (BTV-10) , BTV serotype 17 (BTV-17), or both BTV serotypes and maintained at different temperatures (20°C, 25°C, or 30°C). Every other day, *C. sonorensis* were collected and processed for BTV qRT-PCR to track virogenesis over time. Co-infected *C. sonorensis* collected were processed for BTV plaque-isolation (a technique to visualize replicating virus units). The

complete genotypes of isolated plaque progeny were determined using shotgun next-generation sequencing. Results indicate that *C. sonorensis* maintained at warmer temperatures had productive virogenesis earlier in infection than *C. sonorenesis* held at cooler temperatures. However, *C. sonorensis* maintained at cooler temperatures had longer mean survival times. Most of the plaque progeny virus genotypes aligned with parental serotype BTV-17, while a few plaques had both parental serotypes represented. While warmer temperatures may accelerate virogensis for earlier potential transmission, there is a trade-off with *C. sonorensis* mean survival times. Most of the plaque progeny genotypes aligned with BTV-17 indicating that it may be the more fit serotype in the *C. sonorensis* system.

To further explore why the majority of progeny virus aligned with BTV-17, the second aim was to evaluate if different coinfection ratios of BTV-10 and BTV-17 affect progeny virus genotype outcomes. In prior *in vitro* BTV coinfection and modeling studies, progeny genotypes were dominated by the parental strain with the higher initial multiplicity of infection. To recapitulate this in an *in vivo* model, *C. sonorensis* were fed a blood meal containing BTV-10, BTV-17, or both BTV strains with contributing titers of BTV-10 ATCC: BTV-17 at either 90:10, 75:25, 50:50, 25:75, or 10:90 ratios. Pools of five midges were collected in triplicate every other day and processed for pan BTV qRT-PCR to track virogenesis over time. Day ten post-infection midges were collected in pools of ten and processed for plaque isolation and propagation. The complete genotypes of isolated plaques were identified using shotgun next-generation sequencing. Plaque progeny virus genotyping demonstrated an overall trend of progeny virus aligning with the parental serotype with the higher titer. However, a few plaques at a subset of co-infection ratios demonstrated reassortant genotypes with patterns that were suggestive of preferred segment

combination. While the parental serotype with the higher contributing titer may have more representation in progeny virus, reassortment events can provide genetic diversity.

As reassortment between BTV-10 and BTV-17 was infrequent, it was conjectured that the two parental BTV serotypes did not routinely coinfect the same cells. Thus, the third aim was to determine extent of dissemination and characterize tropism of BTV coinfection in *C. sonorensis*. In situ hybridization approach was employ using the RNAscope® platform to detect patterns of BTV infection in histologic cross sections of coinfected *C. sonorensis*. Upon assessment by microscopy, mosaic patterns in which serotypes did not often overlap, suggest that coinfection at the cellular level may not be abundant with these two serotypes. This could be a consequence of superinfection exclusion. Understanding BTV coinfection and its biological consequences will add an important dimension to the modeling of viral evolution and emergence.

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"Here I was not only troubled by a cloud of stinging midges,
but far more by the doubts of my mind"
– Robert Louis Stevenson: Kidnapped.

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CHAPTER 1 – INTRODUCTION

Introduction

Studies of infectious disease outbreaks have generated awareness that the epidemiology and transmission of these diseases are rapidly changing in concert with climate change. Three closely related orbiviruses (Family *Sedoreoviridae*) of veterinary significance exemplify this concerning pattern: bluetongue virus (BTV), epizootic hemorrhagic disease virus (EHDV), and African horse sickness virus (AHSV).[1,2] Outbreaks of disease with the aforementioned viruses can have devastating effects including hemorrhagic disease for the animal host and economic loss for stakeholders due to production deficits and trade restrictions. These three orbiviruses are recognized by the World Organization for Animal Health and have shared attributes that are significant to their evolution and transmission, including: 1) possession of segmented, double-stranded RNA (dsRNA) that permits evolution via the phenomenon of reassortment; 2) transmission by insect vectors (the *Culicoides* biting midge; Diptera, Ceratopogonidae); and 3) notable expansion into new geographical regions.[1–6] Thus, research findings and insights garnered from one of these viruses of high consequence can have shared implications and applications. The introduction of this dissertation will focus on BTV, the prototype *Orbivirus*, and addresses the following: 1) bluetongue disease (BT) in the ruminant host; 2) BTV transmission via the *Culicoides* biting midge; 3) BTV structure and replication; 4) evolution of BTV; and 5) BTV reassortment requirements in the context of climate variability.

Bluetongue Disease in the Ruminant Host

Disease consistent with BTV infection was first described in 1876 in South Africa, though presumably BTV had been occurring since the late 1700s when European sheep breeds

where introduced to the region.[7,8] Initially the disease was termed "epizootic catarrh" or simply "fever," but due to its association with cyanosis of the tongue, is now known as "bluetongue."[9,10] In French literature, the disease is referred to as 'la fièvre catarrhale ovine' (not be confused with la fièvre catarrhale maligne, a disease caused by ovine herpesvirus-2).[2] While BTV can infect a wide range of ruminant species, sheep are a particularly susceptible host. In addition, there has been documentation of clinical BT in a variety of mammalian hosts including cattle, deer, goats, and alpacas.[11–14] Experimental infection of dromedary camels and American bison demonstrated viremia (i.e., systemic circulation of virus in the blood), but no clinical signs of disease, suggesting these ruminants may serve a role as reservoirs of BTV.[15,16]

Pathogenesis in the ruminant host is initiated by inoculation of BTV from infected *Culicoides* via a cutaneous bite. Inflammation secondary to salivary proteins and trauma from bites recruits leukocytes permissible to BTV infection which then act as a conduit for virus transportation to local lymph nodes for initial replication.[17,18] BTV then disseminates via primary viremia with tropism for mononuclear phagocytic cells such as macrophages, endothelial cells, and lymphocytes.[18,19] Importantly, BTV can tightly adhere to the exterior of red blood cells and remain infectious for approximately 60 days in some hosts with a detectable presence of RNA for up to 7 months.[20–23] BTV tropism for endothelial cells results in the infection of highly vascular tissues. As BTV egresses from mammalian cells in a lytic manner, this is pathologically manifested as widespread vasculitis and edema (often occurring in the lungs), pericardial effusion, facial swelling, ulcerations in the oral cavity (resulting in the early name of "soremuzzle" in the United States), hyperemia of the coronary band, along with hemorrhage, coagulopathies, and fever.[1,18,24,25]

Bluetongue virus infections can range from subclinical to severe and are impacted by previous BTV exposure, virulence of the infecting strain, individual immunological status, and presence of opportunistic infections. Clinically infected animals may demonstrate respiratory distress, difficulty ambulating, reluctance to eat due to oral pain, and wool break due to fever interfering with appropriate wool growth.[24,26] There have been reports of reproductive complications including abortions and testicular degeneration.[27–29] Together, these sequelae may lead to decreased weight gain, poor wool quality, and herd reproduction losses that impact overall livestock production.[1,26]

Control of BTV can be attempted via vaccination. BTV strains serially passaged through sheep several times that consequently became attenuated were used as vaccinations in the early 1900s. [30,31] It was noted that ruminants gained protection against the specific strain used to create the vaccine, however not against other strains. This precipitated the understanding that several different BTV serotypes are in circulation. The serotype of BTV is defined by the host's antibody immune response to the viral protein 2 (VP2) outer capsid protein. These antibodies are serotype specific and don't confer protection to other BTV serotypes.[32,33] Currently there are over 29 BTV serotypes recognized worldwide.[34–38] Indeed, there is concern for incursions of novel BTV serotypes into endemic regions, as novel serotypes often cause severe disease compared to enzootic serotypes due to lack of preexisting protective antibodies for the novel serotype.[39–41] Furthermore, modified-live BTV vaccines have demonstrated reassortment with wild strains while the more expensive inactivated vaccines only provide a transient duration of protective immunity which exemplifies the need to develop innovative vaccine platforms.[42–45]

BTV Transmission via the *Culicoides* Biting Midge

The insect vector culpable for biological transmission of BTV is the hematophagous *Culicoides* biting midge. Du Toit initially demonstrated *Culicoides* harbored infective BTV and AHSV via inoculations of susceptible mammalian species with homogenized wild *Culicoides imicola.*[4] *Culicoides*' role in BTV transmission was substantiated by Du Toit when he demonstrated sheep became infected with BTV via the bite of infected midges. While there are greater than 1300 species of *Culicoides* biting midges, approximately 30 species have been implicated in the transmission of BTV.[46] Different *Culicoides* spp. can exhibit variation in competency (in the context of "vector competency" referring to the innate ability of the invertebrate vector to become infected and transmit a pathogen) for different serotypes of BTV.[47,48] While *Culicoides* are the principal mode of BTV transmission, there are examples of rare serotypes with direct or vertical transmission capabilities in ruminant hosts.[18] Nevertheless, *Culicoides* have several key features that characterize their role as insect vectors including physical characteristics, life traits, and ecology.[49]

Physical characteristics

Culicoides are very small compared to their mosquito vector counterparts. Most *Culicoides* have a body length of 3 mm or shorter, with the main North American species, *Culicoides sonorensis*, having an average body length of 1.5 mm. Their small size is reflected in several colloquial names, including "midges," "no-seeums," and "punkies." *Culicoides* implement a telmophagy (pool-feeding) approach to blood feeding through the use of rasping or chewing mouthparts. The mouthparts are used to cut through the epidermis of the mammalian host and they imbibe the resulting pool of blood.[49] The virus particles present in the insect saliva enter the wound and infect the mammalian host. Some species of *Culicoides* have a saliva protease that alters infectivity of BTV particles by cleavage of the VP2 outer shell protein. VP2

cleavage in this manner produces a more infectious subviral particle. This mechanism has been suggested in some *Culicoides* spp. as an explanation for increased competency (*C. sonorensis*) for BTV as compared to others (*C. nubeculosus*).[50]

Life traits

Though variable, adult *Culicoides* are estimated to live 1-3 weeks.[49] After mating, females of most *Culicoides* spp. require a blood meal to produce viable eggs (anautogenous). If the blood meal is obtained from a BTV infected host, the virus may replicate and disseminate to the midge salivary glands, making subsequent blood meals potentially infective to the next ruminant host. The time from imbibing an infectious blood meal to becoming infective is termed the extrinsic incubation period (EIP) and typically requires 7-10 days.[49] Although *Culicoides* facilitate transmission of BTV to the ruminant host, horizontal transmission between *Culicoides* and transovarial transmission of BTV in the midge has not been observed in laboratory or surveillance studies in the wild.[49,51,52] Thus, BTV transmission is mostly perpetuated by the alternating host cycle between competent *Culicoides* biting midges and susceptible ruminant hosts. Many *Culicoides* are considered non-discriminatory feeders, which makes for an intricate transmission network in the context of vertebrate host diversity.[53–55]

Ecology

The physical characteristics and life traits of *Culicoides* dictate their requisite ecologic parameters, including access to moist environments and appropriate ambient temperatures. For most *Culicoides* spp., ovideposition and larval development require a wet environment. However, optimal or preferred semiaquatic environments are species dependent and vary from farm lagoons (e.g. *C. sonorensis*), dung pats (e.g. *C. brevitarsis*), moist organic soil substrates with less free standing water (e.g. *C. imicola*), and ephemeral bison wallows.[56,57] In addition to a

moist environment for population perpetuation, *Culicoides* have optimal ambient temperature ranges for development and functionality.[56,58]

The habitat requirements of BTV competent *Culicoides* along with presence of BTV was previously documented to occur between geographical latitudes 50°N and 35°S. Seasonality of *Culicoides* and BTV detection similarly parallel optimal conditions with tropical climates exhibiting active BTV transmission yearlong in fluctuation with monsoon seasons. Temperate regions experience outbreaks in the summer and fall seasons with cessation in the winter months.[56] However, with the occurrence of climate change, midge vector presence and BTV outbreaks have been extending beyond previously documented ranges. This includes extensions further north and south as well as occurring earlier in the summer and later into the fall.[59–61]

In addition to orbiviruses, *Culicoides* also transmit bovine ephemeral fever virus, Schmallenberg virus, Akabane virus, and the emerging zoonotic Oropouche virus.[62] Recently, midges have also been recognized as a potential vector for the parasite *Leishmania martiniquensis*.[63] Despite their integral role in disease transmission, *Culicoides* are often cited as one of the least understood arthropod vectors. The challenges in developing research colonies, as well as their small size, have been major impediments for thorough investigations.[49,64] As vectors of several consequential infectious agents, it is incumbent to develop research technologies and strategies that will facilitate further studies of *Culicoides* midges.

BTV Structure and Replication

BTV was confirmed as the etiological agent of BT in 1905.[7,8] Taxonomically, it is assigned by the ICTV (International Committee on Taxonomy of Viruses) to order *Reovirales*, family *Sedaroviridae*, and genus *Orbivirus*.[65] It is a non-enveloped virus with a bi-layered viral capsid that encloses dsRNA made up of 10 individual segments.[66,67] The first segment encodes

for VP1, which is the RNA-dependent RNA polymerase.[68] Segment 2 encodes for VP2, an outer shell protein that mediates entry and confers the antibody response in the ruminant host.[69,70] While VP2 defines the BTV serotype, it is important to be cognizant of potential diversity in the nine remaining segments. BTV is identified by serotype, but there can be many different strains within a serotype. VP3, which is a component of the inner capsid, is encoded by segment 3.[67,71] VP4, a viral capping protein, is encoded by segment 4.[68] Segment 5 encodes for NS1, which assists in viral translation and formation of microtubules in the host cell.[72,73] Segment 6 encodes for VP5, a complimentary outer shell protein, that assists the release of the BTV inner core into the cytoplasm from the late endosome via membrane fusion.[74,75] VP7, an inner shell protein, is encoded by segment 7.[67,71] Segment 8 encodes for NS2, which aids in translation and assembly of the virus by formation of a viral inclusion body (VIB) and mobilization of BTV RNA and proteins for virion construction.[72,76] Segment 9 encodes for VP6, the RNA helicase.[67] An additional protein, NS4, is encoded by segment 9 in an overlapping reading frame which has a role in eluding the host antiviral response via interferon antagonism.[77,78] Segment 10 encodes for NS3 and NS3a, which facilitates viral egress and is mandatory for viral replication in midges.[66,79,80] Bioinformatic analyses posit the existence of an NS5 protein encoded by a second open reading frame on segment 10.[81] NS5 has recently been detected and was demonstrated to interact with host single-stranded RNA (ssRNA) and DNA, suggesting that it modulates the host cell response.

BTV replication commences with attachment to the host cell by VP2. While a definitive cognate receptor has not been identified, VP2 has demonstrated binding to sialic acid, suggesting that the putative receptor molecule is most likely a glycoprotein.[67,82,83] Cellular entry is achieved via clathrin-mediated endocytosis.[74,75] Once within an endosome, uncoating occurs and the virus

core is released into the cytoplasm via confirmational changes induced in VP5. BTV dsRNA is sequestered from early host detection via replication of positive-sense RNA within the sheltered virus core. Eventually, pores are formed when structural arrangements in VP3 and VP7 are induced secondary to the accumulation of magnesium and NTPs.[71,75] Positive-sense RNA templates are then transcribed into viral proteins in the cytoplasm. The formation of protective VIBs provide concentrated locations for progeny virus replication and assembly.[72,76] When the virus core and its components (including all 10 genomic segments) are assembled, virions exit the VIB and obtain outer shell proteins VP2 and VP5.[67,73] In the mammalian host cell, egress of BTV has been identified as mostly lytic, while in the insect vector, nonlytic egress is presumed to occur due to limited cytopathic effect observed in these cells.[66]

BTV Evolution

BTV evolution is intrinsically complex due to inherent characteristics that can influence its genetic trajectory. Characteristics include: 1) an alternating host transmission cycle between mammalian and insect hosts; 2) mutations (e.g., genetic drift) in dsRNA viruses; and 3) segmented genome capable of reassortment. These characteristics provide a framework that supports the intricate balance between genetic stability and genetic diversity that perpetuates transmission and modulates evolution. Consequences of BTV evolution include shifts in mammalian and insect host infectivity, alterations in transmission dynamics, and changes in pathogenicity. Thus, knowledge of BTV evolutionary mechanisms is fundamental to understanding its transmission and subsequent ramifications.

Alternating host cycle

A salient pressure on BTV evolution is that it infects insect and mammalian hosts in alternating host cycles, also known as host switching. It has been proposed that arboviruses with

alternating host cycles require stable genomes and lower mutations rates than non-vector transmitted RNA viruses in order to sustain fitness between the disparate vertebrate and non-vertebrate host systems.[84–86] A West Nile virus study found a low proportion of non-synonymous mutations and non-synonymous/synonymous substitutions per site in both interhost and intrahost populations.[84] This indicates constraints in genetic variation which is suggestive of purifying (negative) selection despite the inherent increased mutation rate of RNA viruses.[84] Indeed, the quelling of nonsynonymous genetic variation interhost is a signature of purifying selection and has been reflected in studies of several different arboviruses.[86,87]

Cell culture provides a convenient *in vitro* platform to simulate the alternating host cycle for evaluation of genetic variations in virus populations. Several cell culture studies of alternate passaging of arboviruses on mammalian and insect cells are supportive of purifying selection. A Rift Valley fever virus (RVFV) study employing alternating passages on BHK-21 (baby hamster kidney) and Aag2 (*Aedes aegypti*) cells did not demonstrate appreciable genetic changes.[88] When serially passaged on only BHK-21 or Aag2 cells, genetic changes in RVFV were readily detectable during subsequent passages on Aag2 cells. In a study by Kopanke et al., BTV was alternately and serially passaged on bovine pulmonary artery endothelial cells and CuVaW3 (derived from *Culicoides* embryo*s*) cells. Both the alternating and serial passaging experiments demonstrated modest genetic variation across all passages.[89] Granted, variations in these findings may be due to different selection pressures specific to cell types employed. This is exemplified in other *in vitro* BTV studies that found more genetic diversity in BTV passaged serially in *Culicoides* cell lines compared to vertebrate cell lines.[90,91] In these studies, different *Culicoides* (KC cells derived from *Culicoides* larva) and vertebrate (BHK and Vero) cells lines were used in the studies, and this may explain some of the observed discrepancies.

Though initially informative, *in vitro* studies do not replicate the complexities of selective pressures present during *in vivo* studies. Complexities include challenges from the immune system, bottlenecks associated with transmission, and virus navigation of different cell types at dissemination barriers. Bonneau et al. performed *in vivo* BTV alternating host studies with sheep, *Culicoides*, and cattle that tracked the genetic sequences for the segments that encoded VP2 and NS3/NS3a through the transmission cycle.[87] Overall, genetic diversity present throughout the transmission cycle was underwhelming, which is consistent with purifying selection. However, the study did capture a founder effect event in which a genetic variant was amplified in a pool of *Culicoides* after feeding on infected sheep. This illustrates the persistent tension between the genetic stability sustained by the alternating host cycle and the mechanisms promoting genetic flexibility required to adjust to new or altered environments.[84,92]

Mutation in dsRNA viruses

Paradoxical to the genetic stabilization of the alternating host cycle, arboviruses do require an element of genetic diversity to adapt to divergent host environments. This may be accomplished by the generation of quasispecies that support genetic plasticity necessary for swift adaption to different environemments.[92–95] Quasispecies is a "swarm" or population of genetically related variants/mutants produced because the RNA dependent RNA polymerase has inferior proofreading, but rapid replication speeds, compared to their DNA polymerase counterparts[84,92,96–98] This can result in progeny virus populations with numerous low-frequency mutations. The spawning of quasispecies gives RNA viruses access to a diverse repertoire of mutants which has been implicated in the ability for RNA viruses to adapt to and infiltrate new environments and overcome challenges imposed by host immunity and therapeutic interventions.[99–101]

However, not all mutations are beneficial to virus fitness, and excessive mutation rates in RNA viruses have been predicted to potentially be detrimental to virus survival.[102,103] The double-stranded structure of BTV's RNA genome may elevate its genome stability by increasing fidelity in replication. Indeed, BTV nucleotide substitution rates have been calculated at a mean rate of approximately 0.5-7 x 10^{-4} nucleotide substitutions per site per year which is even lower than other (single-stranded RNA) arboviruses.[85,104,105]

Reassortment

Reassortment is a mechanism for quickly establishing substantial changes to a virus genotype. The phenomenon of reassortment occurs when at least two different parental strains of the same segmented virus species coinfect the same cell with resulting progeny virus obtaining segment contributions from the different parental strains.[106–108] Reassortment is a hallmark of BTV evolution and considered a principal driver of BTV genetic diversity and adaptive fitness.[108,109] Accumulations of non-beneficial mutations, a process known as Muller's Ratchet, may also be efficiently replaced with reassortant events.[110,111] Notable virus families with segmented genomes include *Orthomyxovirdiae*, *Birnaviridae,* and *Picobirnaviridae.*[112–114] Order *Bunyavirales*, in addition to order *Reovirales,* contain examples of segmented viruses that are arboviruses including RVFV, La Crosse virus, and Schmallenberg virus.[115,116] However, Bunyaviruses only have three genomic segments. BTV's 10 segmented genomic dsRNA equates to 1024 different potential combinations between two different strains.

Reassortant viruses have caused devastating infectious disease events throughout history, including multiple influenza pandemics and the 1997 and 1998 outbreak of hemorrhagic fever from Ngari orthobunyavirus.[117–119] Genetic outcomes of reassortment are implicated in spillover events in which new host species can become infected.[120,121] Similarly, BTV reassortment has

exhibited large genotypic changes reflected in phenotypic changes in pathogenicity and infectivity.[108] Readily demonstrated in field isolates, BTV reassortment is often implicated in manifestations of more severe disease.[122,123]

Segmented genomes of BTV may also be a mechanism for regulation of gene expression. A recent study demonstrated that certain BTV segments are more abundant than others and that segment frequencies differed between the ruminant and midge host. Specifically segments 1, 7, and 10 had differing abundance between ruminant and midge. Varying abundances of segments can also have implications on evolutionary rates between segments.[124] Auxiliary benefits of a segmented genome include demonstrated stability and rapid replication which further highlights the complex role of reassortment in virus evolution.[125]

Reassortment Requirements in Context of Climate Change

Evidence from field surveillance studies implicates reassortment in the rapid and extensive expansion of BTV.[44,122,126,127] However, for successful reassortment to occur, specific requirements must be met: 1) sympatry in which two different strains of BTV are in circulation at the same time in the same geographical range; 2) ecology in which the two strains must infect the same host species; 3) cellular coinfection in which the two strain must be able to infect the same cell; 4) segment packaging in which the two strains are able to package each other's segments into the same virion; 5) replicative compatibility in which the two strains are able to replicate each other's segments; and 6) fitness in which the reassortant progeny virus are able to compete with the parental strains.[116,128]

Many of the requirements for reassortment can be affected by altered temperatures. The intersection between reassortment and climate change include effects on the geographical and temporal range of *Culicoides*, *Culicoides* and ruminant life traits, and virus biology. Numerous

studies investigating novel incursions and spread of BTV postulate that climate change is an important driver to disease emergence.[61,129–131] Projected temperatures from earth system models have been featured as the sole driver in various orbivirus projections.[59,129] Indeed, climate change models have corresponded with BTV expansion observations and surveillance.[59,130–133] For example, the sudden expansion of BTV into Northern Europe was preceded by an unusually warm summer in 2006.[60,131,134] Thus, exploration of reassortment requirements for BTV in the context of climate change is escalating in relevance.

Sympatry

Sympatry of circulating BTV serotypes has a complex and multifactorial history. Originally, BTV was presumed to be confined to the African continent. However, in the mid-1900s, BTV emerged beyond the African continent to the Iberian Peninsula, Cyprus, and the United States.[135–138] As BTV infiltrated new environments, and was subjected to different selective pressures, this gave rise to genetic diversification of genotypes and subsequent serotypes.[139,140] Currently, BTV has a global presence, apart from Antarctica, and continues to expand beyond its canonical geographic range.[12] Additionally, there has been an increase of incursions of novel serotypes into endemic areas which creates more opportunities for reassortment between different serotypes.[126,141–143]

Europe provides a striking example of the speed in which several BTV serotypes became established and the devastation they caused. Prior to 1998, BT was categorized as an exotic disease in Europe, but by 2005 there were five different BTV serotypes (1, 2, 4, 9, 16) circulating in Southern Europe. In 2006, BTV serotype 8 (BTV-8) entered Europe, presumably from Northern Africa, causing a serious epizootic outbreak and extended northward.[26,141] In addition

to disease in sheep, cattle alarmingly demonstrated clinical infection and losses were estimated to be over 150 million €.[24,26,144,145]

BTV-3 and BTV-6 incursions throughout the United States, that until recently were considered non-endemic serotypes to the United States, also demonstrate how sympatry and subsequent reassortment facilitate geographic expansion.[41,127] Prior to 2022, only BTV serotypes 2, 10, 11, 13, and 17 were classified as endemic to the United States by the United States Department of Agriculture Animal & Plant Health Inspection Service (USDA APHIS).[127,146]

BTV-3 was initially detected in Florida in 1999. Surveillance post 2006 demonstrate its detection further west including Mississippi, Louisiana, Arkansas, South Dakota, and Texas.[127,147,148] Comparing genetic sequences of BTV-3 isolates endemic to Central America and the Caribbean, United States' BTV-3 isolates acquired most segments from serotypes that are endemic to the US. This supports the hypothesis that reassortment between non-endemic serotypes and endemic serotypes enable non-endemic serotypes to become established in new regions. Similarly, BTV-6 was intermittently detected in Florida since 2006, but was isolated for the first time in Colorado in 2020 from sheep clinically presenting with BTV infection.[41,149] In 2021, BTV surveillance of sheep and cattle in Colorado regularly detected BTV-6, which has also been implicated in seven deceased mule deer.[41]

The fluctuation of BTV serotypes isolated in the United States persuaded USDA APHIS to amend how endemic and non-endemic strains were classified. In 2022, APHIS reclassified BTV serotypes in the United States as "established" (3, 6, 10, 11, 12, 13, 17), "reported" (1, 2, 5, 9, 14, 15, 18, 19, 22, 24), or "not reported" (4, 7, 8, 16, 20, 21, 23, 25, 26) to provide the flexibility needed to address the ever changing BTV serotype landscape.[150]

Predictably, climate change can increase the number of sympatric BTV serotypes in circulation.[131,151] This is mainly attributed to the expanding geographical range for *Culicoides* vectors that are competent for different serotypes. In Europe, the establishment of BT in the late 1990s was ascribed to a climate change driven expansion of *Culicoides imicola* from Northern Africa into the Mediterranean basin.[151] The incursion of *C. imicola* infected with BTV exposed *Culicoides* spp. indigenous to Europe, such as Palearctic species complexes (*Culicoides obsoletus* and *Culicoides pulicaris*), to BTV that subsequently exhibited vector competence in what is referred to as the "baton effect." The "baton effect" is when a *Culicoides* spp. competent for BTV introduces BTV or non-endemic BTV serotype into a new region via geographical expansion that overlaps with another *Culicoides* spp. that is also competent.[152] In due course, BT outbreaks occurred in northern regions were *C. imicola* are not present, but Palearctic species thrive.[151,153–155] In a similar manner, *Culicoides insignis,* the main vector of BTV in the Neotropical region, has noticeably expanded their geographic range throughout Southeast United States and is implicated in introducing BTV serotypes such as BTV-6 and BTV-3 into the United States. BTV-6 and BTV-3 have been detected in Western United States where *Culicoides sonorensis* is the primary vector.[156–158]

An opposing theory to climate change vector-driven emergence of BTV reassortment hypothesizes that *C. imicola* did expand into the Mediterranean basin due to climate change, although this movement occurred long ago in the Late Pleistocene and Holocene (approximately 17,000 years ago). This suggests that the forces behind BTV incursions are more related to viral genomic evolution than vector expansion.[159] Indeed, as the "baton effect" theory illustrates, several mechanisms are probably involved in the increasing number of circulating serotypes in the same region. This most likely includes geographical expansion of vectors due to climate

change and virus genome evolution (including reassortment) which may facilitate competency in a new host or vector species, or otherwise alter competency in an established host or vector.

Ecology

BTV reassortment events have been recognized in both ruminants and *Culicoides* hosts.[160–164] The host must be susceptible for two or more serotypes and support viral replication and transmission. Vector competency is a specific term that embodies the innate ability of the invertebrate vector to become infected and transmit a pathogen.[48] The transmission dynamics ecology of BTV is complex as different hosts can exhibit varying susceptibilities and competencies for different BTV serotypes.[108] This restricts reassortment to serotypes with the capacity to coinfect the same host. As an illustration, a coinfection study between BTV-10 and BTV-2 in colony-reared *C. sonorenesis* demonstrated that the midges from this colony were not competent for BTV-2. Therefore, all progeny virus aligned with BTV-10 with no reassortment events.[165] The parameters that define vector competency for different BTV serotypes are poorly characterized and the relationship between BTV genotype and phenotype of infectivity requires further study. Additionally, infected *Culicoides* and ruminant hosts must be in sympatric habitats to each other, with consideration for adequate population density, to support the alternating host cycle.

Detection of two different BTV serotypes in the ruminant host have been captured in experimental and surveillance studies.[162,163,166] Evidence of natural coinfections within the ruminant host is particularly compelling as it demonstrates the satisfaction of sympatry and ecology reassortment requirements. When BTV-5 was detected for the first time India in 2010, one of the diagnostic samples from a ruminant had presence of both BTV-5 and BTV-1 segment 2. The presence of BTV-5 was believed to have added to the severity of disease in the outbreak

as the ruminants were naïve for that serotype.[166] In the US, natural coinfection of individual cattle with BTV-11 and BTV-17 has been detected in a sentinel cattle herd.[163] As subclinical infections often occur in cattle and goats, they could serve as inconspicuous reservoirs for reassortment to occur, thus highlighting the importance of sentinel surveillance programs. Experimentally, reassortment has been recapitulated in a heifer that was infected with two electrophoretically different strains of BTV-11 with recovered virus comprising of one of the parental strains along with reassorted virus.[164] Coinfection studies using BTV-1 and BTV-8 have been performed in sheep and calves, and demonstrated that both serotypes infected the host simultaneously. However evaluation for the presence of reassortant progeny virus was not completed.[167,168]

Culicoides BTV coinfections have been investigated in the laboratory setting; however, natural coinfection events in the field of the midge are difficult to capture due to the logistics of screening a large numbers of insects with short lifespans.[161,169,170] An added challenge is that characterizing vector competency for certain virus strains can be difficult and often fluctuates in response to environmental factors. Even same species vectors from conserved geographic regions can demonstrate a range of competencies, presumably due to microbiome changes from variable environmental exposures.[171] For example, if a mammalian host is receiving antibiotic treatments, the antibiotic could alter the vector microbiome and competence. In fact, one study applied antibiotics to modify *Culicoides* microbiome and detected downstream effects on Schmallenberg virus infection rates.[172,173] Alterations in the environment may influence the vector's ability to replicate and transmit virus which emphasizes the importance of viral genetic diversity in changing environments.

As to whether the vertebrate or invertebrate host is more conducive to generating reassortants, current dogma indicates that reassortment is more likely to occur in the invertebrate host. This is supported by the premise that invertebrates have less stringent immune systems, making them more permissive to infection and subsequent reassortment. Samal et al. conducted BTV coinfection studies investigating reassortment in *Culicoides* and sheep using the same BTV-17 and BTV-10 strains.[160,161] Viral plaques isolated from progeny viruses were classified as reassortants: 5% in the sheep and a range of 7% - 78% in individual *Culicoides*.[160,161] However, a recent *in vitro* study comparing reassortment of *Bunyamwera orthobunyavirus* and *Batai orthobunyavirus* in BHK-21, Aag2, and *Aedes albopictus* (U4.4 immune competent and C6/36 immune deficient) cell lines found reassortment in the mammalian cell line, but not in any of the insect cell lines.[119] The authors noted that their findings contradict current literature supporting more reassortment in insect hosts. However, as knowledge of insect immunology progresses, it is becoming apparent that it is more complex than previously thought which may lead to alterations to current arbovirus paradigms.

Climate change can impact BTV ecology requirements for reassortment by affecting ruminant susceptibility or *Culicoides* competency for BTV. In the veterinary community there is growing recognition that climate change can affect animal health.[174,175] Abrupt changes in the environment, such as increased temperature and drought, can stress the ruminant immune system, making them more susceptible to infection and possibly increase the occurrence of coinfections.[176,177] Climate change can also impact components of BTV ecology including an invertebrate vector population's potential to transmit BTV, a concept known as Vectorial Capacity (VC), which may indirectly increase reassortment potential by augmenting opportunities of coinfection via escalated transmission.

As ectothermic creatures, climate change can have significant effects on *Culicoides* ecology, physiology, and virus replication. This is illustrated by climate change effects on VC, the quantification of vector transmission potential that is calculated by:

$$VC = \frac{ma^2bp^N}{-\ln(p)}$$

where 'm' is density of vector, 'a' is biting rate, 'b' is vector competence, 'N' is extrinsic incubation period, 'p' is daily probability of vector survival.[178,179] Increase in ambient temperature has been demonstrated to affect all components of vectorial capacity.[180]

Optimal climes support the success of species reproduction and increase density of the vector populations. An example specific to *Culicoides* is larval development and oogenesis rates accelerate at higher temperatures.[56,180–182] Moreover, the host-biting rate is linked to the rate of oogenesis as egg development requires a bloodmeal (most *Culicoides* are nonautogenous).[182,183] Mechanistic models suggest that the viral EIP decreases and vector competency increases as environmental temperatures increase.[48,59]

Several studies support evidence that virogenesis is positively correlated with temperature in the vector.[165,184] *Culicoides* exposed to BTV became infected faster and produced peak level of virus detection sooner at higher temperatures than those kept in lower temperature. Similarly, laboratory detection of EHDV occurred sooner in *Culicoides* housed at 30°C as compared to those housed at 25°C or 20°C.[185] The causation for this is likely multifactorial and include effects of temperature on the vector as well as on the virus itself. Viral nucleic acid synthesis and viral protein synthesis increase with temperature, which can be explained by the generally increased rate of biochemical reactions (modelled by the Arrhenius equation in chemical kinetics) increasing exponentially with temperature. This has been supported in work by Scholtessick and Rott that measured rates of avian influenza virus hemagglutinin and

neuraminidase synthesis along with the enzymatic activity of its RNA dependent RNA polymerase at different temperatures.[186]

Temperature effects on vector physiology can alter their response to viral infections. Ambient temperature impacts the insect immune response, including expression of defensin and cecropin antimicrobial proteins.[187,188] Cooler temperatures are linked to upregulation of melanin which assists with cold acclimatization and innate immunity.[189,190] Increased temperatures have also been associated with increased dissemination of virus through vector barriers which facilitate virus reaching the salivary glands and ensuing transmission to subsequent ruminant hosts.[53,191,192] Identified BTV dissemination barriers of *C. sonorensis* include the mesenteron infection barrier (prevents BTV infection of midgut), mesenteron escape barrier (limits BTV to gut cells), and a general dissemination barrier (prevents BTV in the haemocoel from infecting other tissues).[193] Currently, a salivary gland infection barrier and a salivary gland escape barrier for BTV have not been recognized in *Culicoides*.[193] Increased feeding frequency due to the stretching of the basement membrane in the midgut has been implicated in facilitating passage of pathogens through the mesenteron escape barrier.[194] Thus, it could be conjectured that increased feeding and biting rates secondary to increased temperatures may also facilitate virus dissemination.

In combination with the above, increased temperatures may reduce vectorial capacity due to decreased probability of vector survival. This is strongly supported in several temperature studies with laboratory-reared *Culicoides*.[165,195,196] Thus, there is a balance between survivorship and virogenesis and there is likely a temperature that optimizes both.[192]

Cellular coinfection

In addition to coinfection of the same host, reassortment requires that the parental viruses infect the same cell. Fundamental to the cellular coinfection requirement is the necessity of similar viral tropisms and the phenomenon of superinfection exclusion. As aforementioned, in the ruminant host, BTV has a tropism for endothelial cells and lymphocytes, as well as mononuclear phagocytes such as macrophages.[18,19] In *Culicoides,* the extent of BTV tropism remains largely unknown and is challenging to assess due to the vector's small size.[64] For both the ruminant and *Culicoides* host, the cellular receptors for BTV are poorly characterized. Superinfection exclusion occurs when an active cellular viral infection prevents a second infection from another related or unrelated virus. Exclusionary infection in this matter would effectively prohibit reassortment. This has been documented in several different viruses including BTV.[112,169,197–200] Currently, it is thought that viruses that are not closely related may be able to overcome superinfection exclusion.[197] A recent development in understanding BTV mechanisms of superinfection exclusion is the extracellular vesicle (EV) release of BTV virions. While these vesicles are efficient at initiating infections and superinfection exclusion, they are less able to overcome superinfection exclusions than their free-virus counterparts.[200] An *in vitro* study showed extracellular vesicles were the majority of virus secreted in both mammalian and insect cells. As BTV egress in insect cells has been characterized as non-lytic, EVs add exciting dimensions to BTV reassortment dynamic including the following questions: 1) Is EV or free virus more prevalent in the insect host? 2) If the insect host has more intact cells due to non-lytic egress, are there more cells to accommodate superinfection and subsequent reassortment? and 3) Do EVs containing different parental facilitates reassortment?

Another element to superinfection exclusion is that it may be time-bound, a theory that has been explored in asynchronous infection studies. In BTV coinfection studies using *in vivo*

Culicoides and *in vitro* Vero cell models, asynchronous infections were separated by varying time intervals.[169,170] Superinfection exclusion was established seven days after initial infection in the *Culicoides* and after four hours in the Vero cells. Thus, if sufficient time passes from the initial infection to exposure to a second strain, the vector may be refractory to the second infection.

Once a cell is coinfected with two different parental strains, it remains unclear how the two strains interact within the cell. Current understanding is that BTV replicates in sequestered VIBs; however, recent work has been able to visualize VIB dynamics in the cell and has captured VIB fusion events in the cell. These fusion events may elucidate how different parental strains connect within the cell to achieve reassortment.[201]

Although studies are limited, it could be extrapolated that climate change affects the occurrence of cellular coinfection. Thermodynamic models support stronger binding of arboviruses, including BTV, to host cells at higher temperatures.[202] Additionally, reassortment requirements for coinfection intersect with ecological considerations in that alterations in host density and circulating BTV strains secondary to climate change may inform the time interval a vector is exposed to two different strains. If the time interval for exposure to a second virus is shortened, coinfection may occur before the advent of superinfection exclusion.

Segment Packaging

The mechanisms by which segmented viruses adequately package a complete set of genomic segments into a forming virion is not fully understood. Studies have indicated that BTV undergoes a selective packaging process that ensures that all ten BTV segments are appropriately contained within each virion.[66] This may be accomplished by controlled, inter-segment interactions via conserved untranslated regions (UTRs) of the 3' and 5' end of each segment. For

example, segment 10's 3' UTR is required to initiate this process and confer secondary conformation structures to achieve correct assemblage.[203] The remaining segments then aggregate in coordinated order: segments 7-9, segments 4-6, and then segments 1-3.[67] Thus far, temperature effects on segment packaging have not been interrogated, although it would be interesting to investigate if warmer temperatures increased binding interactions between segments or if increased kinetics would reduce the fidelity of correct packaging.

Replicative compatibility

Replicative compatibility refers to the ability of the viral proteins to cohesively undergo viral replication. A study that exemplifies this concept by Patton et al. demonstrated that rotaviruses (order *Sedoreoviridae*) required a complementary VP2 to assist VP1 (the RNA dependent RNA polymerase) for effective viral replication.[204] In the case of reassortment, if a segment in a progeny strain encodes a protein that is not conducive to replication, that progeny virus cannot be sustained. Characterization of replicative compatibility in BTV has not been described in the literature at the time of writing this dissertation.

In principal, replicative compatibility would suggest that the need for compatible virus components would favor reassortment between viruses that are more closely related.[116] *In vitro* experiments with bunyamwera group viruses and Hanta virus demonstrated that closely related viruses were more likely to produce viable reassortant progeny.[205,206] This is in contrast to superinfection mechanism in cellular coinfection that favors viruses that are not as closely related.[197] The dichotomous genetic predilections of replicative compatibility and superinfection exclusion further illustrate the perpetual balance between genetic stability and diversity.[116] It is uncertain at this time if climate change would impact replicative compatibility.

Fitness

Viral fitness is the capacity of a virus to generate infectious progeny. The capacity for the virus to replicate and transmit can fluctuate depending on the given conditions.[207] In the context of reassortment, reassortant progeny virus must have sufficient fitness to compete with their parental strains. Fitness competition between strains was illustrated in experimental infection of calves with BTV serotypes 1 and 8.[167] In single infections, both BTV-1 and BTV-8 produced robust viraemia and antibody response. In the case of coinfection with both serotypes, BTV-8 dominated in PCR serotype assays. This was an indication that either BTV-8 dominated the coinfection, or if there was reassortment between the two strains, BTV-8 segment 2 was favored. In a different study, reassortant progeny virus demonstrated their fitness dominance in an *in vitro* coinfection also using BTV-1 and BTV-8. After four passages, all isolated plaques were determined to be reassortants and no pure parental genotype was recovered.[110]

As discussed previously, climate change undoubtably can alter the conditions within the ruminant or vector host including host gene expression, immunity, and the microbiome, which may require a different fitness landscape for the virus to be successful. Thus, reassortants that have more optimal fitness properties in warmer climes may usurp the founding parental strains.

Conclusion

BTV is a virus of veterinary significance that is capable of large genetic changes via reassortment. Requirements for reassortment may be accelerated in the context of climate change. These affects range from molecular, to organismal, to environmental levels. In summary, increasing ambient temperatures influences the range expansion of *Culicoides* which can transport novel BTV serotypes into new geographic regions. This begets the sympatric circulation of novel serotypes. Warmer seasons can affect vector ecology and augment vectorial capacity, facilitating higher infection incidence of vertebrate hosts. Additionally, temperature

may stress animal health with consequences for host susceptibility and vector competency. Temperature has demonstrated the ability to increase kinetics of molecular interactions that accelerates virogenesis and strengthens receptor binding. This may have unexplored consequences on cellular coinfection, segment packaging, and replicative compatibility. Finally, new reassortants may have increased fitness for the changing environment. The emergence of novel BTV reassortants further perpetuates the infiltration of BTV serotypes into new regions.

BTV evolution mechanisms maintain a balance between genetic stability and diversity. Alternating host cycles, double-stranded genomes, and requirements for segment packaging and replicative compatibility support genetic stability. The ability to reassort, the RNA genome, and mechanism of superinfection exclusion support genetic diversity. Thus, it is important to continue the surveillance of this virus system and be prepared for new important incursions.

There are several challenges to studying BTV, including the small size of its *Culicoides* vector, its presence at the fluctuating domestic-wildlife interface, range of genetic diversity, and its unique genomic properties. These challenges are highlighted by knowledge gaps in characterizing the genetics of circulating viruses, profiles for ruminant host susceptibility and vector competence, and molecular mechanisms of virus reassortment. Advances in next-generation sequencing and processing of unconventional dsRNA viruses will be critical for enhancing approaches to molecular and surveillance studies. Applying and optimizing platforms such as in situ hybridization techniques may assist with understating virus-vector dynamics by facilitating the visualization of cell tropism and dissemination in such a small insect. BTV has inherent characteristics that highlight the complex intersections of reassortment requirements in the context of climate change. These intersections may further propel the potential for reassortment opportunities and viral emergence which urgently necessitates further investigation.

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CHAPTER 2 – EVALUATING TEMPERATURE EFFECTS ON BLUETONGUE VIRUS SEROTYPE 10 AND 17 COINFECTION IN *CULICOIDES SONORENSIS*

Introduction

Climate change has been implicated as a determinant in the emergence of diseases caused by arboviruses.[1–5] One such arbovirus is bluetongue virus (BTV), the causative agent of bluetongue disease (BT) that has produced devastating epizootics in domestic and wild ruminant populations. BTV is transmitted to susceptible ruminant species by the *Culicoides* biting midge (Diptera: Ceratopogonidae) with the geographic range of BTV defined by the presence of competent *Culicoides* species.[6,7] In concurrence with climate change, the geographic range of *Culicoides* and BTV is expanding. In 2006, BTV serotype 8 (BTV-8) entered Europe and progressed northward causing substantial illness in livestock with losses that were estimated to be over 150 million €.[8–12] Increased vector distribution secondary to climate change is a prominent explanation for these incursions. However, temperature effects on the virus itself may have ramifications on BTV evolution and transmission.[13–16]

BTV is the prototype virus of the genus *Orbivirus* in the order *Reovirales*, family *Sedaroviridae,* and is non-enveloped with a segmented double-stranded RNA genome. [17] It has ten genomic segments with segment 2 encoding for the viral protein 2 (VP2) that confers the serological response in susceptible hosts. Current BTV nomenclature identifies the virus by different serotypes which corresponds to the serological response to VP2.[18–20] There are over 29 recognized BTV serotypes. [21] It important to recognize that while BTV is traditionally identified by serotype, there is additional diversity in the remaining nine genomic segments. As a virus with a segmented genome, reassortment is an important mechanism for BTV evolution.[22,23]

Reassortment occurs when two or more parental virus serotypes exchange genomic segments that result in progeny strains with novel segment combinations. Previous studies of BTV, and of the related epizootic hemorrhagic disease virus (EHDV) orbivirus, demonstrated that virogenesis increases in the *Culicoides* vector at warmer temperatures. As well, *Culicoides* fed BTV or EHDV bloodmeals became infected quicker at higher temperatures compared to those held at lower temperatures.[13,14,24] However, the impact of temperature on reassortment has not been characterized.

Given the abundance of BTV serotypes, coupled with the potential for exchange between all ten genomic segments, reassortment has the potential to generate a multitude of novel BTV genotypes. Granted, some requirements for reassortment have been identified that may limit the extent to which different parental BTV strains can naturally reassort. In the field, it is necessary for the parental BTV strains to be co-circulating in the same geographic region (sympatric requirement) and they must be able to infect the same species of host (ecological requirement). Additionally, they must coinfect the same cell (coinfection requirement) and be able to package each other's genomic segments (segment packaging requirement) within a single virion.[25] The proteins encoded by the parental viruses must be able to function together to productively replicate progeny virus (replicative compatibility requirement).[26] Finally, after those requirements have been met, progeny reassortants must be able to compete with the parental genotypes (fitness requirement).[27]

Despite the complexity of these requirements, evidence of BTV reassortment is prevalent in experimental and field studies and is predicted to have significant effects on transmission, pathogenicity, and range of host species susceptibility, and therefore requires further investigation.[22,28–33] Many early studies applied electrophoretic analysis to determine progeny

virus genotypes. However, there are few contemporary studies that have investigated reassortment in *Culicoides* using the highly sensitive next-generation sequencing (NGS) platform. As *Culicoides* are ectothermic, ambient temperatures of BTV infected *Culicoides* may affect *Culicoides* life traits and virus replication. Therefore, this study aims to compare how ambient temperatures of 20°C, 25°C, or 30°C affect *Culicoides* survival, virogenesis (i.e., viral progeny production), and potential reassortment in *Culicoides sonorensis,* the main vector of BTV in the United States, during coinfection with BTV-10 and BTV-17.[6] Additionally, we assess virogenesis and *Culicoides* survival during single infections of BTV-10 and BTV-17 to identify if virogenesis of the parental serotype corresponds to progeny virus genotypes and coinfection trends. Identifying factors that affect BTV evolution and insect vector survivorship will further inform our understanding of BTV transmission and emergence of novel strains.

Materials and Methods

Viruses, virus titration, and cell culture,

The BTV strains employed in this study were selected because of their endemicity to the United States and their relative genomic differences by pairwise identity (Table 2.1). BTV-10 California 1952 (BTV-10) (Bluetongue virus, type 10, strain 8, ATCC® VR-187™) was procured from ATCC and passaged eight times on BHK 21 cells. [34] BTV-17 Colorado 2018 (BTV-17) was isolated on CuVaW3 cells from BTV-positive whole blood from a naturally infected sheep with clinical signs and passaged one time on BHK 21 cells. [35] As both of these serotypes were isolated in the United States, it is anticipated that the colony *C. sonorensis* will exhibit competence for both serotypes. Whole genome sequences of each virus were performed previously by our lab and are available on GenBank (BTV-10 GenBank Accession MW456747-MW456756; BTV-17 GenBank Accession OQ798198-OQ798207).[35,36]

Table 2.1 – Nucleotide Pairwise Identity of BTV-10 & BTV-17 Genomic Segments

Segment	Protein encoded	% Pairwise Identity
Seg-1	VP1	96.1
Seg-2	VP2	68.8
Seg-3	VP3	97.3
Seg-4	VP4	96.3
Seg-5	NS1	96.9
Seg-6	VP5	79.5
Seg-7	VP7	95.8
Seg-8	NS2	96.2
Seg-9	VP6/NS4	96.9
Seg-10	NS3/NS3A/NS5	81.7

Infectious titer for BTV-10 and BTV-17 was determined by 50% tissue culture infectious dose ($TCID_{50}$) in triplicate with negative controls and calculated using the Reed-Muench method.[37] In brief, eight 10-fold dilutions were prepared for each virus in Eagle's Minimum Essential Medium with Earle's salts and without L-glutamine (EMEM) (Corning Cell-Gro). Fifty µL of each virus dilution were added sequentially onto a 96-well plate in addition to 50 µl EMEM for sample wells and 100 µL for control wells. Approximately 1.55×10^4 BHK 21 cells were added to each well. The inoculated plates were incubated at 37°C and 5% CO_2 for 96 hours. After incubation, crystal violet solution was introduced into the wells for staining of viable cells.[36]

BHK 21 cells were perpetuated in BHK 21 media comprising of EMEM, 10% insect cell culture tested fetal bovine serum (certified, heat inactivated, US origin from Sigma Aldrich), 10% tryptose phosphate broth (Sigma Aldrich), and 1% penicillin streptomycin (10,000 U/ml). The cells were incubated at 37°C with 5% CO_2 and passaged every three to four days when culture flasks were 80-90% confluent. [36]

Culicodes sonorensis infection and maintenance

Culicoides sonorensis were supplied by the Arthropod-Borne Animal Diseases Research Unit (United States Department of Agriculture, Agricultural Research service, Manhattan, KS), specifically from the AK colony that originated from the field in Owyhee Co., Idaho, August 1973.[38] *C. sonorensis* were shipped overnight by air at one day of age and held at 25°C, with a 12:12 hour light cycle, and 10% (w/v) sugar water *ad libitum* for two days before infection studies commenced.

Defibrinated sheep blood (Hemostat Laboratories, Dixon, CA) was screened for BTV virus and antibody by pan BTV qRT-PCR and the Bluetongue cELISA kit (VMRD, Pullman WA). Single infection blood meals were prepared by spiking defibrinated sheep blood with respective BTV serotypes to produce a viral titer of 1.0×10^5 median $TCID_{50}$/ mL. Blood meals containing both serotypes were prepared by spiking defibrinated sheep blood to produce equal titers of 5.0×10^4 $TCID_{50}$ / mL of BTV-10 and BTV-17 for a total viral titer of 1.0×10^5 $TCID_{50}$/ mL. Infection groups included single infections of BTV-10, single infections of BTV-17, coinfections of BTV-10 and BTV-17, and uninfected blood for negative controls. *C. sonorensis* were fed assigned infection blood meal treatments for a duration of one hour and 30 minutes via mosquito glass feeders through parafilm membranes with blood heated to 37°C by a water bath.

After receiving the bloodmeal, the *C. sonorensis* were chilled for four minutes at -20°C and placed on a modified chill table to facilitate sorting and collection of engorged females from each infection group into containers to be housed in 20°C, 25°C, or 30°C incubators (Table 2.2). There were nine different treatment groups that included BTV-10 and BTV- 7 coinfection groups maintained at 20°C, 25°C, or 30°C; BTV-10 single infection group maintained at 20°C, 25°C, or 30°C; and BTV-17 single infection group maintained at 20°C, 25°C, or 30°C. Additionally, there

were uninfected negative controls maintained at 25°C for pan BTV qRT-PCR analysis and at 20°C, 25°C or 30°C for survival studies. Immediately after feeding, five to ten blood-fed females from each infection group were collected to assess uptake of virus via pan BTV qRT-PCR. For the duration of the infection experiment, *C. sonorensis* were maintained in respective incubators with a 12:12 hour light cycle and housed in containers of non-treated paper tube containers (Rigid Paper Tube Corporation, Wayne, NJ) topped with sheer pantyhose over the lid for access to feeding and air exchange. Sugar water prepared at 10% (w/v) continued to be provided *ad libitum* and uninfected blood meals were provided every four days for 30 minutes. Humidity and temperatures of incubators were monitored daily.

Due to constraints in numbers of *C. sonorensis* that could be procured and housed at the same time, different experiments of the study were performed at separate times. First, the *C. sonorensis* BTV-10 and BTV-17 co-infection experiment was performed (Experiment 1). Next, BTV-10 single infection and BTV-17 single infection of *C. sonorensis* in addition to survival studies were performed (Experiment 2). An additional survival study was performed due to limited number of *C. sonorensis* (Experiment 3). The parameters for the experiments are outlined in Table 2.2 for the qRT-PCR and plaque assays and Table 2.3 for the survival assays. The same BTV stocks were employed for each experiment and negatives controls were included for each experiment in the study.

Table 2.2 - *C. sonorensis* BTV Infections for PCR and Plaque Assays in Experiments 1 & 2

	BTV-10			BTV - 17			BTV - 10 & 17			Negative	
Temperature	20°C	25°C	30°C	20°C	25°C	30°C	20°C	25°C	30°C	25°C	25°C
Experiment ID	2	2	2	2	2	2	1	1	1	1	2
Number of Midges per container	n = 150	n = 151	n = 150	n = 151	n = 153	n = 156	n = 400	n = 407	n = 407	n = 103	n= 150
	n = 150	n = 194	n = 128	n = 194	n = 183	n = 191	n = 425	n = 247	n = 339		n = 69
Mean Bloodmeal BTV Titer ($TCID_{50}$/mL)	1.0×10^5	1.0×10^5	1.0×10^5	1.0×10^5	1.0×10^5	1.0×10^5	BTV-10: 5.0×10^4 BTV-17: 5.0×10^4	BTV-10: 5.0×10^4 BTV-17: 5.0×10^4	BTV-10: 5.0×10^4 BTV-17: 5.0×10^4	–	–

C. sonornesis survival studies

C. sonorensis survival studies were performed for each temperature and infection group combination including negative controls. *C. sonorensis* from each representative infection group were housed 50 midges per container in duplicate. Daily survival counts were performed and recorded (Table 2.3).

Table 2.3 - *C. sonorensis* BTV Infections for Survival Assays in Experiments 2 & 3

	BTV-10			BTV - 17			BTV - 10 & 17			Negative		
Temperature	20°C	25°C	30°C	20°C	25°C	30°C	20°C	25°C	30°C	20°C	25°C	30°C
Experiment ID	2	2	2	2	2	2	2 and 3	3	3	2 and 3	2 and 3	2 and 3
Survival groups	n = 50 in duplicate	n = 50 in duplicate	n = 50 in duplicate	n = 50 in duplicate	n = 50 in duplicate	n = 50 in duplicate	n = 50 in duplicate	n = 50 in duplicate	n = 50 in duplicate	n = 50 in duplicate	n = 50 in duplicate	n = 50 in duplicate
Mean Bloodmeal BTV Titer ($TCID_{50}$/mL)	1.0×10^5	1.0×10^5	1.0×10^5	1.0×10^5	1.0×10^5	1.0×10^5	BTV-10: 5.0×10^4 BTV-17: 5.0×10^4	BTV-10: 5.0×10^4 BTV-17: 5.0×10^4	BTV-10: 5.0×10^4 BTV-17: 5.0×10^4	–	–	–

C. sonorensis collections

To longitudinally evaluate virogenesis via pan BTV qRT-PCR, five *C. sonorensis* were collected in triplicate from each infection group at each temperature every other day. For plaque assays to assess progeny genotypes, pools of ten *C. sonorensis* from the coinfection groups at

each temperature were collected in triplicate on day 3, 7, 11, 15, and 19.[24] *C. sonorensis* were collected from each group until none remained.

Coinfection plaque assays

The *C. sonorensis* pools collected for plaque assays were immediately processed for plaque isolation. Five hundred µL of EMEM was added to each pool of ten *C. sonorensis* and then manually homogenized with a sterile pestle, vortexed, and centrifuged. The supernatant was sterile-filtered with a syringe filter (0.22 µM millex-GV syringe filter, MilliporeSigma, Burlington, MA). Fifty µL of the supernatant was reserved for extraction for downstream serotype specific qRT-PCR and sequencing. The remaining supernatant was used to make dilutions of 1:2, $1:10^1$, $1:10^2$, :$1:10^3$, and $1:10^4$ in EMEM for plaque assays. Six-well plates with a confluent monolayer of BHK 21 cells were used for the plaque assays. Confluency was achieved by seeding BHK 21 cells (1.0×10^5 cells/well) with BHK 21 media, as described above, and incubated at 37°C and 5% CO_2 for 48 hours prior to the plaque assays. Immediately before inoculation with *C. sonorensis* dilutions, each well was washed with 0.5 mL PBS pH 7.4. Five hundred µL of each *C. sonorensis* dilution was instilled into separate designated wells including a negative control well for each 6-well plate. The inoculated 6-well plates were placed in 37°C incubators for one hour and gently rocked every 15 minutes to facilitate even dispersion of the virus. The inoculum was gently aspirated off the cell monolayers and each well washed with 0.5 mL PBS pH 7.4. Two mL of an overlay mixture containing 3:1 BHK 21 media: 2% agarose in Earle's Buffered Salt Solution (EBSS) warmed to 40°C was applied to each well. The 6-well plates were incubated at 37°C and observed twice daily for visualization of plaques. When plaques first appeared (approximately 48 hours after incubation) a second overlay of one mL/well of 3:1 BHK 21 media: 2% agarose in EBSS with 0.1% neutral red stain was added and

the plates were incubated at 37°C until 8-24 hours after the second overlay when visible plaques were apparent.

For plaque propagation, individual plaques from the 6-well plates were harvested using a 1000 µL barrier filter pipette tip and each plaque was placed in an individual well of the 48-well plate that contained BHK 21 cells (4.65 X 10^4 cells/well). The 48-well plates with the harvested plaques were incubated at 37°C and observed twice daily for cytopathic effects (which usually occurred after three - four days). When 3 - 4 + cytopathic effect was observed in a well, the propagated virus plaque was collected and stored at -80°C until extraction and library preparation for NGS.

Nucleic acid extraction and DNase treatment

The pools of five *C. sonorensis* collected for pan BTV qRT-PCR were organized and processed for extraction. All pools of *C. sonorensis* collected in triplicate on days 4, 8, 12, and 16 post infection were processed for qRT-PCR. Two of the three pools were processed from *C. sonorensis* collected on days 2, 6, 10, 14, 18 days post infection with unprocessed pools reserved for potential individual analysis. For processing, each pool was manually homogenized with a sterile pestle in 250 µL EMEM, vortexed, and centrifuged. Fifty µL of the supernatant was transferred to a 96-Deep-well King Fisher plate for nucleic acid extraction. Nucleic acid extraction was performed using the MagMAX Pathogen RNA/DNA Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's low-cell content protocol and processed on the KingFisher Flex robot (Thermo Fisher, Waltham, MA) with 90 µL elution volume. After extraction, samples were treated with DNase 1, RNase free (Thermo Fisher). Two µL of DNase 1 and 2 µL of 10X buffer were added to 12 µL of extracted sample and incubated at 37°C for 30

minutes. To inactivate the DNase, 2 µL of EDTA was then added to each sample and incubated at 65°C for ten minutes. [24]

The propagated plaques and the supernatants from the homogenized *C. sonorensis* pools that were used to isolate the plaques were extracted using the MagMAX Pathogen RNA/DNA kit according to the low-cell protocol with 50 µL input, however they were processed manually with a 45 µL elution volume. The extracts were then treated with TURBO DNA-*free*™ kit (Invitrogen). In brief, the TURBO DNA- *free*™ treatment protocol was 2-4 U of DNase in 0.1 volume of DNase buffer per sample and incubated for 30 minutes at 37°C. For DNAse inactivation, 8 µL of inactivation reagent was added with 5 minutes of incubation at room temperature. The samples were centrifuged (10,000 x g for 1.5 min) and supernatant collected. The supernatant was treated with LiCl for a 2.0 M final concentration for selective precipitation of single-stranded RNA. The samples were incubated for 16 hours at 4°C and then centrifuged (18,000 x g at 4°C for 20 minutes). Supernatant was collected and remaining salt was removed using a 1.25x MagMAX Pathogen RNA/DNA Kit clean-up. [24]

Pan BTV and cox1 qRT-PCR assay

Each extracted, DNased *C. sonorensis* pool was processed in duplicate for the detection of BTV with a universal pan BTV qRT-PCR that targets BTV segment 10, as described by Kopanke et al., using half-reaction volumes of the SuperScript III One-step qRT-PCR kit (Thermo Fisher). [24,39] Additionally, qRT-PCR of *Culicoides* mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) was performed on each sample in duplicate to normalize for extraction efficiency variation. FAM-based probe (3'FAM- TGAATACTT/ZEN/CCTCCTTCTCTTTCTT - 3IABkFQ/5', Integrated DNA Technologies, Coralville, IA) was designed previously by our lab based on GenBank sequences of this gene using Geneious v.10.2.2.[24,39–42] Similar as the pan

BTV qRT-PCR protocol, SuperScript III One-step qRT-PCR reagents and volumes were used, except samples did not undergo the initial 95°C five minute denaturation step with the primers.[39] Positive and negative controls for both BTV and *Culicoides cox*1 were run with each PCR plate and a no-reverse transcriptase (no-RT) control was run to ensure that DNAse treatment was effective (ie, no detection of *cox*1 in absence of reverse transcriptase). Normalization of BTV Ct values calculated for each sample was accomplished using the ΔCt method based on mean Ct values for BTV and *cox1* (ie. Pan BTV Ct – *cox1* Ct).[24]

To detect if there was presence of both BTV-10 and BTV-17 segment 2 in pools of *C. sonorensis* used for plaque propagation, a BTV-10 and BTV-17 serotype specific qRT-PCR was performed as described by Maan et al.[43] A Ct value less than 36 was considered positive for detection.

Library preparation and whole genome sequencing

To assess the genotype of the ten genomic BTV segments for each propagated plaque, shotgun whole genome sequencing was performed on the extracted, DNAsed, and LiCl treated samples. With each library preparation and sequencing run, negative water controls as well as positive controls BTV-10, BTV-17, and BTV-10:BTV-17 at 50:50 were performed. The NEBNext® Ultra II Directional RNA Library Prep Kit of for Illumina® (New England BioLabs Inc., Ipswich, MA, USA) was used to prepare sample libraries. Section 4 Protocols (for use with Purified mRNA or rRNA depleted RNA) were performed with the following modifications for double-stranded RNA: 1) RNA fragmentation at 90°C for one minute and 2) first strand cDNA synthesis reaction was processed at ten minutes at 25°C, 30 minutes at 42°C, 15 minutes at 70°C, with a hold at 4°C. For sample identification, NEBNext Multiplex Oligos for Illumina (96 Index Primers) were used according to manufacturer's instructions. Libraries were assessed for DNA

quality and concentration with Qubit high sensitivity DNA reagents on the Qubit 2.0 fluorometer (Thermo Fisher, Waltham, MA, USA) and High Sensitivity D1000 DNA screentape on the TapeStation 2200 or 4150 instrument.

Pools of approximately 60 samples were processed at a time and subsequently size-selected for inserts from 300-900 base pairs with base pair size fractionation on a 1.5% agarose gel. The desired region of the gel was excised and was reextracted using the QIAquick Gel Extraction Kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). A final bead-clean up using NEBNext® Ultra II Directional RNA Library Prep Kit of for Illumina® Section 4.9 protocol was performed to optimize nucleic acid concentration. Afterwards, the pool was analyzed with the Qubit fluorometer and TapeStation instruments as described above and then quantified with the KAPA Library Quantification kit (KAPA Biosystems) according to kit instructions. To perform paired-end sequencing (1 x 50), the NextSeq 500 sequencer instrument (Illumina, USA) was used with the NextSeq 500/550 Mid Output Kit v2.5 (300 cycles).

BTV analysis pipeline and bioinformatics

Libraries were demultiplexed and analyzed with the stenglein-lab/ btv_segment_table pipeline (https://github.com/stenglein-lab/btv_segment_table). This pipeline uses codes and infrastructure developed by the n-f core community. [44,45] The objective of this pipeline is to quantify the number of parental strain virus reads that map to each of the ten bluetongue virus genomic segments in a sample to facilitate assessment of genotype and reassortment events. Cutadapt performed trimmings of low quality sequences, bases, and adapter sequences. Cd-hit-est collapsed duplicate read pairs. Bowtie 2 performed read alignments to the reference sequences (BTV-10 and BTV-17) that we provided. The pipeline was run with default parameters with the exception of the BTV Reference Sequences. FASTA files of BTV-10 and

BTV-17 reference sequences used in this study were formatted and executed with the --refseq_fasta command line option.

Statistical Analysis

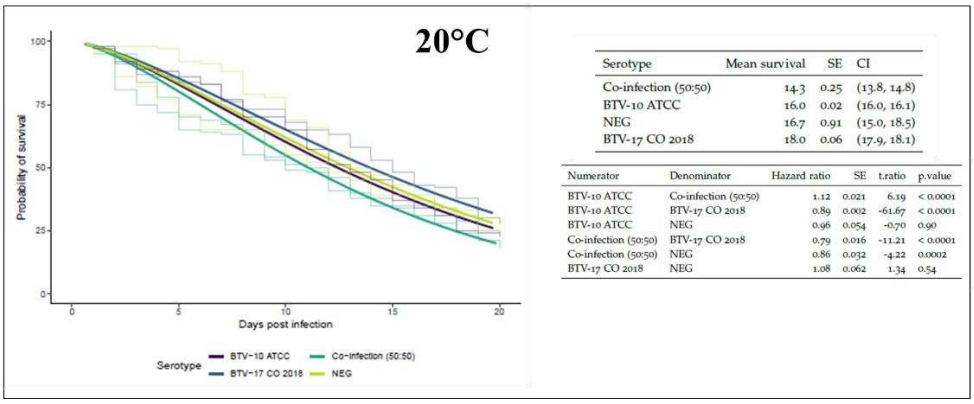
For survival analysis, parametric survival regression models were fit to each temperature separately with a default Weibull distribution and robust sandwich error calculation.[46,47] Additionally, a Kaplan-Meier step curve was included. Mean survival times were calculated by survival regression with standard error and 95% confidence interval. Comparisons between infection groups of survival odds is expressed as the ratio. The standard error of the ratio, statistic and p-value were also determined.

Notably, the shorter survival times of the *C. sonorensis* at warmer temperatures impeded comparisons of virogensis between temperatures groups. Additionally, BTV-10 had several pools of *C. sonorensis* with BTV not detected. To accommodate comparisons between infection groups, the undetermined Ct values were imputed using multivariate imputation by chained equation with a Bayesian linear regression method.[48–50] To account for outliers, robust linear methods were employed.[51] For the 20°C temperature groups a third-degree polynomial was fit to the Ct values, and for both the 25°C and 30°C temperature group a second-degree polynomial was fit to the Ct value. Pairwise comparisons of the linear portions between infection groups within a temperature treatment were made with a Tukey HSD adjustment. Estimates of differences in linear trends, standard error of the difference, z-ratio statistic and p-values were determined. Statistics for qRT-PCR and survival analysis were conducted in open source software R version 4.2.1. Bar graphs and heat maps of plaque genotypes were accomplished with GraphPad Prism 8.1.0 (321).

Results

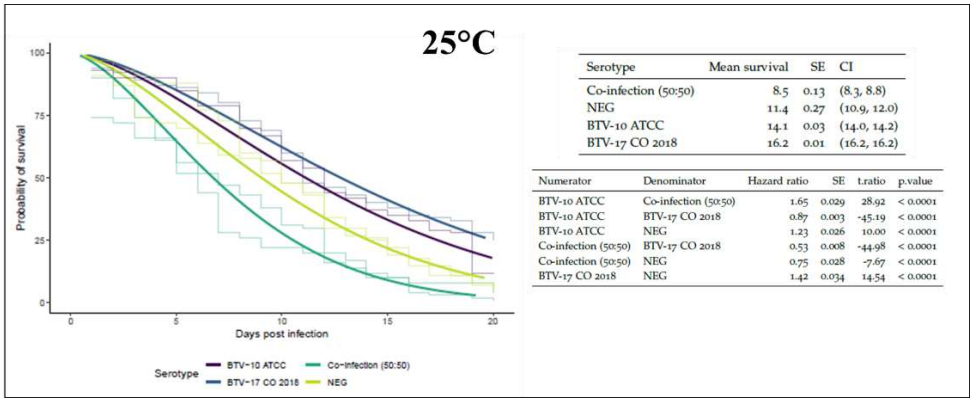
Temperature affects C. sonorensis mean survival

Survival studies were performed for each infection group at each temperature to evaluate if temperature or infection status affected survivability. It is important to consider that the hazard ratios reported may be calculated as significantly different because of small standard errors but might not be different enough for biological meaning (Figure 2.1). In the 20°C temperature group, statistical survival differences were not detected between BTV-17 and the negative control, nor BTV-10 and the negative control (Figure 2.1). In the 25°C temperature group, there was statistical differences between all infection treatments. Notably, the coinfection group had approximately half the probability of survival as the BTV-17 infected group (Figure 2.1). In the 30°C temperature group, there were statistical differences among all infection groups except between the coinfection and the negative control group (Figure 2.1). Across all temperature groups, the coinfection group consistently had the lowest mean survival time and BTV-17 had the highest mean survival time (Figure 2.1). Importantly, across all infection groups, mean survival decreased as ambient temperature increased.



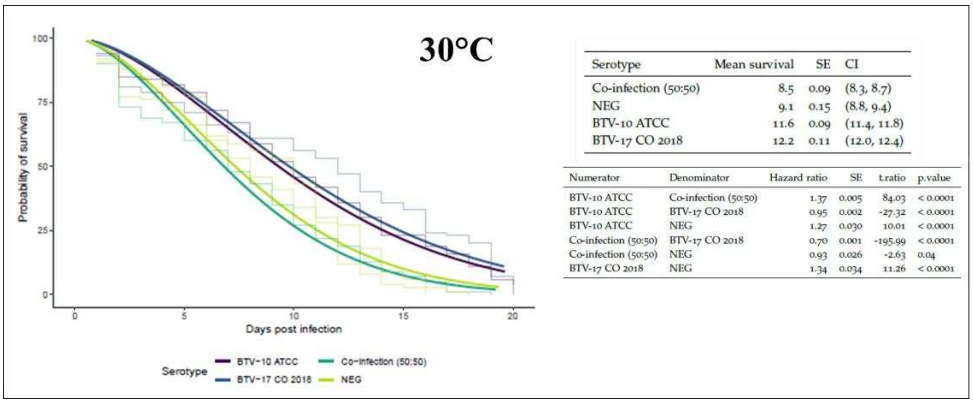
Serotype	Mean survival	SE	CI
Co-infection (50:50)	14.3	0.25	(13.8, 14.8)
BTV-10 ATCC	16.0	0.02	(16.0, 16.1)
NEG	16.7	0.91	(15.0, 18.5)
BTV-17 CO 2018	18.0	0.06	(17.9, 18.1)

Numerator	Denominator	Hazard ratio	SE	t.ratio	p.value
BTV-10 ATCC	Co-infection (50:50)	1.12	0.021	6.19	< 0.0001
BTV-10 ATCC	BTV-17 CO 2018	0.89	0.002	-61.67	< 0.0001
BTV-10 ATCC	NEG	0.96	0.054	-0.70	0.90
Co-infection (50:50)	BTV-17 CO 2018	0.79	0.016	-11.21	< 0.0001
Co-infection (50:50)	NEG	0.86	0.032	-4.22	0.0002
BTV-17 CO 2018	NEG	1.08	0.062	1.34	0.54



Serotype	Mean survival	SE	CI
Co-infection (50:50)	8.5	0.13	(8.3, 8.8)
NEG	11.4	0.27	(10.9, 12.0)
BTV-10 ATCC	14.1	0.03	(14.0, 14.2)
BTV-17 CO 2018	16.2	0.01	(16.2, 16.2)

Numerator	Denominator	Hazard ratio	SE	t.ratio	p.value
BTV-10 ATCC	Co-infection (50:50)	1.65	0.029	28.92	< 0.0001
BTV-10 ATCC	BTV-17 CO 2018	0.87	0.003	-45.19	< 0.0001
BTV-10 ATCC	NEG	1.23	0.026	10.00	< 0.0001
Co-infection (50:50)	BTV-17 CO 2018	0.53	0.008	-44.98	< 0.0001
Co-infection (50:50)	NEG	0.75	0.028	-7.67	< 0.0001
BTV-17 CO 2018	NEG	1.42	0.034	14.54	< 0.0001



Serotype	Mean survival	SE	CI
Co-infection (50:50)	8.5	0.09	(8.3, 8.7)
NEG	9.1	0.15	(8.8, 9.4)
BTV-10 ATCC	11.6	0.09	(11.4, 11.8)
BTV-17 CO 2018	12.2	0.11	(12.0, 12.4)

Numerator	Denominator	Hazard ratio	SE	t.ratio	p.value
BTV-10 ATCC	Co-infection (50:50)	1.37	0.005	84.03	< 0.0001
BTV-10 ATCC	BTV-17 CO 2018	0.95	0.002	-27.32	< 0.0001
BTV-10 ATCC	NEG	1.27	0.030	10.01	< 0.0001
Co-infection (50:50)	BTV-17 CO 2018	0.70	0.001	-195.99	< 0.0001
Co-infection (50:50)	NEG	0.93	0.026	-2.63	0.04
BTV-17 CO 2018	NEG	1.34	0.034	11.26	< 0.0001

Figure 2.1 –Temperature Affects *C. sonorensis* Mean Survival. Plots include all groups from both survival assay trials (experiment 2 and 3) with parametric survival regression models fit to each temperature separately (20°C, 25°C or 30°C) with default Weibull distribution and robust sandwich error calculation. The plots consist of a line for the survival regression curve for each infection group (BTV-10 in purple, BTV-17 in blue, BTV 10:BTV17 coinfection in green, and negative control in yellow) with the y-axis representing probability of survival and x-axis representing days post infection. A Kaplan-Meier step curve is included for each group with a separate step curve for the repeated coinfection and negative control groups from experiment 3. Results are shown in associated tables that provide the mean survival days post infection, standard error (SE), and 95% confidence interval (CI) for individual curves and survival odds (Hazard ratio), SE, statistic (t.ratio), and p-value for comparisons between curves.

BTV-10 and BTV-17 exhibit different proportions of infected pools of C. sonorensis.

BTV was undetectable in several pools of *C. sonorensis* following infection at various ambient temperatures (Figure 2.2). In the 20°C temperature group, there were eight pools of *C. sonorensis* from the BTV-10 single infection group that did not have detectable virus and four pools of *C. sonorensis* from the BTV-17 single infection group that did not have detectable virus. In the 25°C temperature group, there were seven pools of *C. sonorensis* from the BTV-10 single infection group that did not have detectable virus and only one pool of *C. sonorensis* from the BTV-17 single infection group that did not have detectable virus. In the 30°C temperature group, there were five pools of *C. sonorensis* from the BTV-10 single infection group that did not have detectable virus and two pools of *C. sonorensis* from the BTV-17 single infection group that did not have detectable virus. Across all temperatures, BTV was detected in all pools of the coinfection groups (Figure 2.2).

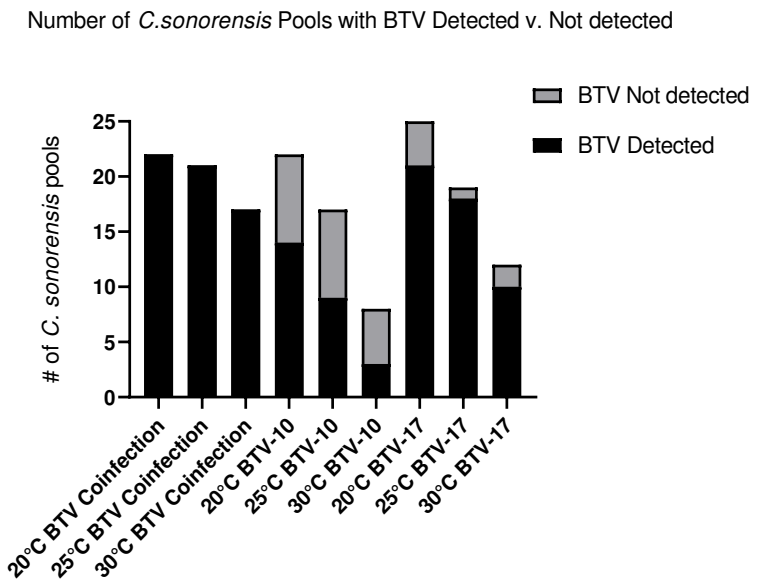
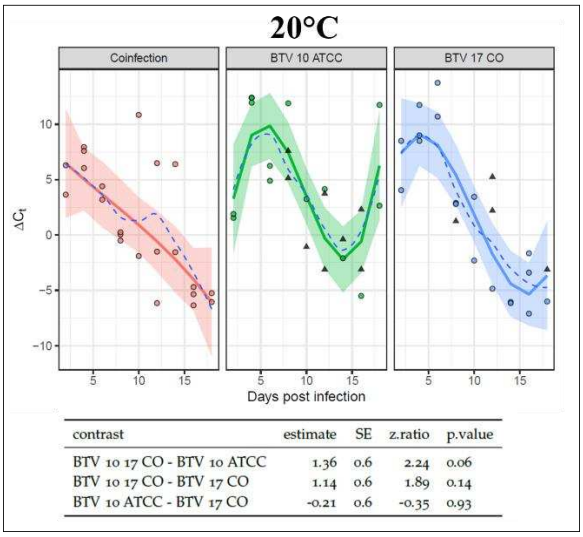


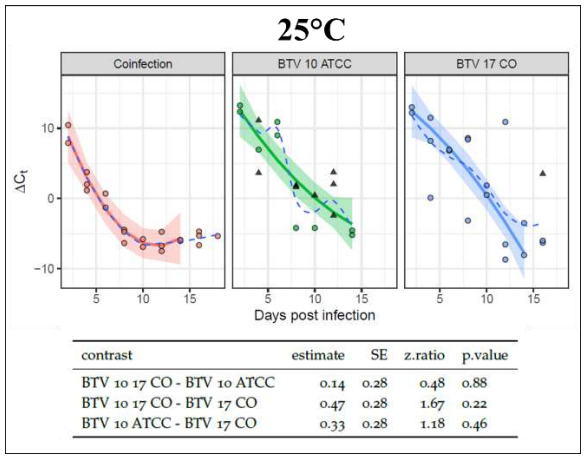
Figure 2.2 – BTV Detected in all Pools of the Coinfection Group. BTV detection was determined by pan BTV qRT-PCR. Black indicates BTV detected and gray indicates BTV not detected.

Temperature affects BTV virogenesis in C. sonorensis

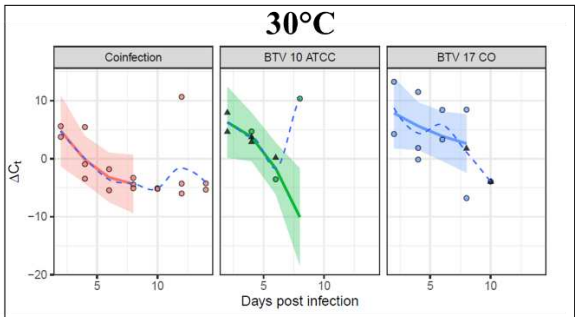
Overall, no significant differences in virogenesis were indicated between infection groups (Figure 2.3). Decreased survivability at warmer temperatures impeded comparisons of virogenesis between some temperature groups. Granted, there were notable trends between temperature groups when performing plaque assays (Table 2.4). At day three post infection, all three pools of *C. sonorensis* reared at 30°C produced plaques; however, only two of the three pools of *C. sonorensis* reared at 25°C produced plaques, and none of the three pools of *C. sonorensis* reared at 20°C produced plaques. *C. sonorensis* pools collected seven days post infection and afterwards produced plaques in all temperature treatments. These findings corroborate previous studies and indicate that warmer temperatures correspond with increased early virogenesis.



contrast	estimate	SE	z.ratio	p.value
BTV 10 17 CO - BTV 10 ATCC	1.36	0.6	2.24	0.06
BTV 10 17 CO - BTV 17 CO	1.14	0.6	1.89	0.14
BTV 10 ATCC - BTV 17 CO	-0.21	0.6	-0.35	0.93



contrast	estimate	SE	z.ratio	p.value
BTV 10 17 CO - BTV 10 ATCC	0.14	0.28	0.48	0.88
BTV 10 17 CO - BTV 17 CO	0.47	0.28	1.67	0.22
BTV 10 ATCC - BTV 17 CO	0.33	0.28	1.18	0.46



contrast	estimate	SE	z.ratio	p.value
BTV 10 17 CO - BTV 10 ATCC	0.14	0.28	0.48	0.88
BTV 10 17 CO - BTV 17 CO	0.47	0.28	1.67	0.22
BTV 10 ATCC - BTV 17 CO	0.33	0.28	1.18	0.46

Figure 2.3 – BTV Virogenesis is Similar across Infection Groups. Due to absence of Ct values for *C. sonorensis* at higher temperatures because of decreased survivability, a linear model was fitted to each temperature (20°C, 25°C or 30°C) separately with robust linear models applied to account for outliers. Some pools did not have detectable BTV and are represented by imputed values using multivariate imputation by chained equation with a Bayesian linear regression method and represented by triangles. A third-degree polynomial was fit to Ct values for the 20°C plot and second-degree polynomials were fit to Ct values for the 25°C and 30°C plots. Solid lines indicate the model curve, the shaded region represents the standard error, and the dash line is a curve based on the mean of the data at each day post infection. Pairwise comparisons were performed on the linear portions of the curves with a Tukey HSD adjustment. Estimates of the differences in linear trends, standard error, z-ratio statistic, and p-value are given for all comparisons. Overall, comparisons of virogensis between infection groups indicated no significant differences. However, due to several of the BTV-10 pools not being infected, comparison by p-values maybe unreliable.

Table 2.4 – Detection of Viable BTV and Serotyping of *C. sonorensis* Pools Used for Plaque Assays. Tables indicate which pools were able to generate viable BTV for plaque isolation assays Additionally, *C. sonorensis* pools were screened for BTV-10 and BTV-17 via serotype specific qRT-PCR. Presence of viable virus and serotype detection are indicated by (+). Absence of viable virus and serotype not detected is indicated by (-). (I) indicates an inconclusive serotype result (Cts > 36). Days where *C. sonorensis* were not available for plaque assay are indicated by (n/a). Additionally, pools of *C. sonorensis* that did not produce viable virus were not screened for BTV-10 or BTV-17 which is indicated by (-). Plaques from pools that had adequate sequences reads that all aligned to BTV-17 were not processed for further sequencing are indicated by (0) in the No. Plaques Sequenced column.

20°C					
			PCR Serotype Detection		
Day Post Infection	Pool	Plaque Assay	BTV-10	BTV-17	No. Plaques Sequenced
3	A	-	n/a	n/a	n/a
	B	-	n/a	n/a	n/a
	C	-	n/a	n/a	n/a
7	A	+	-	+	2
	B	+	-	+	2
	C	+	-	+	2
11	A	+	-	+	2
	B	+	-	-	2
	C	+	-	+	2
15	A	+	I	+	5
	B	+	-	+	2
	C	+	-	+	2
19	A	+	-	+	2
	B	+	-	+	0
	C	+	+	+	10

25°C					
			PCR Serotype Detection		
Day Post Infection	Pool	Plaque Assay	BTV-10	BTV-17	No. Plaques Sequenced
3	A	+	I	+	2
	B	+	-	+	2
	C	-	n/a	n/a	n/a
7	A	+	I	+	5
	B	+	-	+	2
	C	+	-	+	2
11	A	+	-	-	2
	B	+	-	-	2
	C	+	-	+	0
15	A	+	I	+	5
	B	+	-	+	0
	C	+	-	+	2
19	A	n/a	n/a	n/a	n/a
	B	n/a	n/a	n/a	n/a
	C	n/a	n/a	n/a	n/a

30°C					
			PCR Serotype Detection		
Day Post Infection	Pool	Plaque Asssay	BTV-10	BTV-17	No. Plaques Sequenced
3	A	+	-	+	2
	B	+	-	+	2
	C	+	-	+	2
7	A	+	+	+	10
	B	+	-	+	2
	C	+	-	+	2
11	A	+	-	+	2
	B	+	-	+	0
	C	+	-	+	0
15	A	+	-	+	0
	B	n/a	n/a	n/a	n/a
	C	n/a	n/a	n/a	n/a
19	A	n/a	n/a	n/a	n/a
	B	n/a	n/a	n/a	n/a
	C	n/a	n/a	n/a	n/a

BTV-10 and BTV-17 serotype detection in pools of coinfected C. sonorensis

BTV-17 serotype was overwhelmingly represented in *C. sonorensis* pools from the coinfection group (Table 2.4). Plaques propagated from coinfected *C. sonorensis* were prioritized for sequencing by detection of both BTV-10 and BTV-17 via serotype-specific qRT-PCR. One pool from 19 days post infection at 20°C and one pool from seven days post infection at 30°C had qRT-PCR detection for both serotypes. There were four pools that had detectable BTV-17 and were inconclusive for BTV-10. Sequencing of the pools was attempted; however, due to low reads, most of the pools could not be adequately characterized. Six pools did have adequate sequencing reads but only demonstrated hits against BTV-17 across all segments, and so plaques were not sequenced from those pools (Table 2.4). Ten plaques were sequenced from pools that had both serotypes represented by qRT-PCR (both with Ct values of 36 and under). Five plaques

were sequenced from pools that had both serotypes represented, but one serotype had an inconclusive classification (Ct values of 36 and above). Two plaques were sequenced from pools that only had one serotype detected.

Majority of plaques from coinfected C. sonorensis align with BTV-17

For genotype assessment, plaques were classified as having either a BTV-10 genotype, a BTV-17 genotype, a reassortant genotype, or a mixed genotype as determined by sequencing reads. As described by Kopanke et al., if >90% of the reads of a segment aligned to one parental strain and all ten segments aligned with the same parental strain, the plaque was classified as having the genotype of that parental strain (BTV-10 or BTV-17). If >90% of the reads of a segment aligned to one parental strain, but not all segments came from the same parental strain, the plaque was classified as reassortant.[36] Mixed genotypes were plaques that contained at least one segment that aligned to both parental strains (neither parental strain had >90% reads represented in a segment). The 19 day post infection pool at 20°C had four plaques with a mixed genotype. The remainder of the plaques aligned completely with BTV-17 (Figure 2.4). The seven day post infection pool at 30°C had four plaques that aligned completely with the BTV-10 parental strain and only three plaques that aligned with the BTV-17 parental strain. The remaining three plaques represented mixed genotypes. None of the plaques were definitively characterized as reassortants. Plaques sequenced from other *C. sonorensis* pools were completely represented by the BTV-17 genotype (Figure 2.4). The negative and positive BTV-10 and BTV-17 controls used for sequencing indicated that plaque genotypes were appropriately detectable and distinguishable (Appendix 1). Overall, these results demonstrate the complexity (and potential stochasticity) of coinfection.

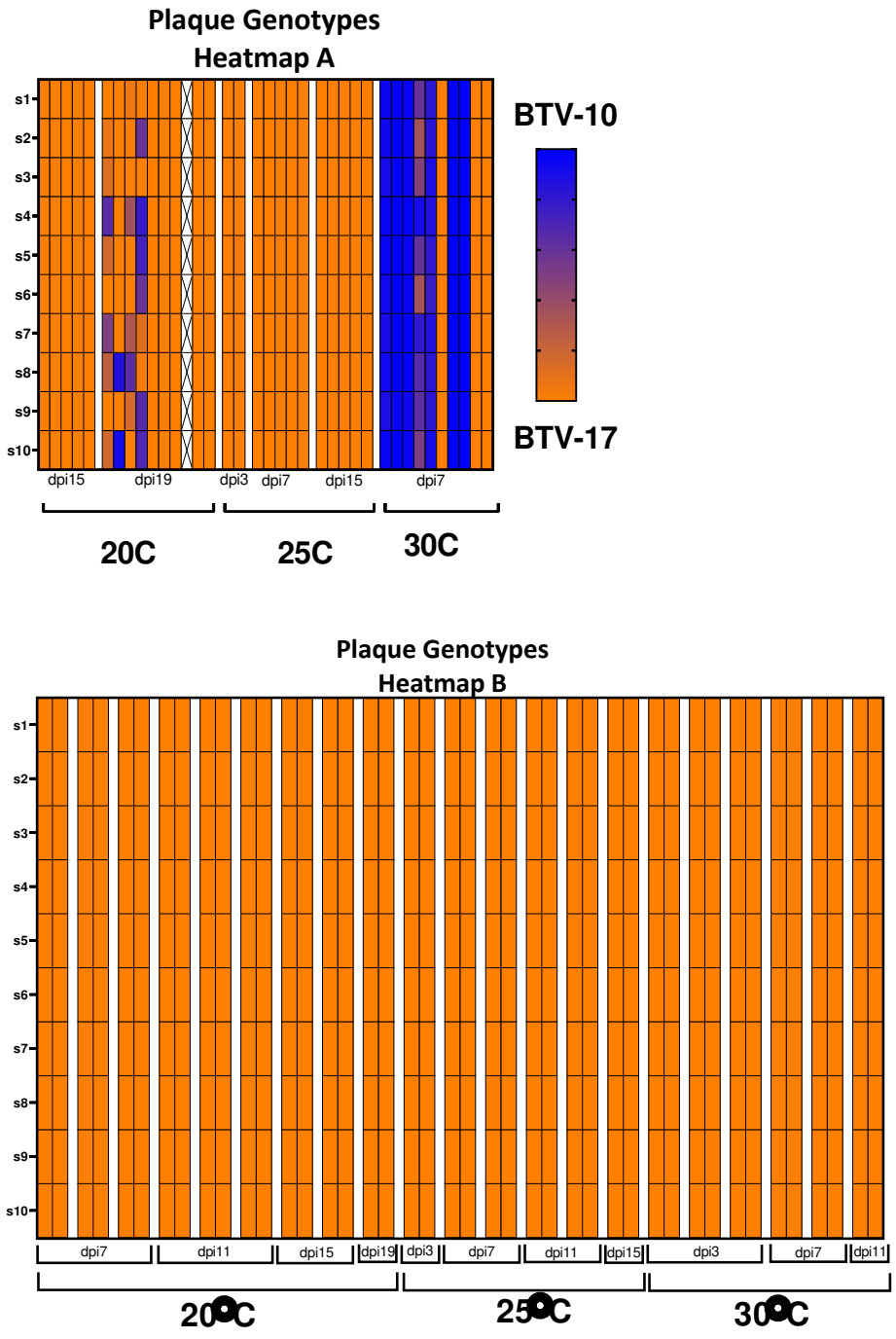


Figure 2.4 – BTV-17 is the Majority Plaque Genotype. Plaque genotypes that were sequenced from coinfected *C. sonorensis* are depicted by heat maps. **Heatmap A)** Represents plaque genotypes from pools that had qRT-PCR detection of both BTV-17 and BTV10 serotypes. **Heatmap B)** Represents plaque genotypes from pools that had qRT-PCR detection of only BTV-17 serotype. Each column on the x -axis represents an individual plaques with pools separated by white margins and labeled by day post infection (dpi). The y -axis represents the ten different genomic segments of BTV. Blue indicates that 100% of the sequencing reads for that segment

aligns with BTV-10. Orange indicates that 100% of the sequencing reads for that segments align with BTV-17. A Color gradient in between blue and orange indicates that the segment had reads from both parental strains. X in a segment indicates insufficient reads.

Discussion

The impetus for this study is the increasing evidence from field and laboratory research that climate change may be accelerating the expansion and emergence of impactful arboviruses such as BTV. Specifically, warming temperatures may coincide with climate change in certain geographic regions. To evaluate how this change might affect factors of BTV transmission and evolution, we investigated temperature effects on *Culicoides* survival, rates of BTV virogenesis, and BTV progeny virus genotype outcomes of *Culicoides* coinfected with BTV-10 and BTV-17.

The first component of this study was to evaluate survivability of the *Culicoides* vector at increasing ambient temperatures. This is an important component of transmission dynamics as the longer an infected vector lives, presumably the more time it has to feed on and infect a susceptible host. Overall, we found that coinfected groups consistently exhibited shorter mean survival times than single and negative infection groups. Kopanke et al. observed a similar trend in *C. sonorensis* housed at 20°C that were exposed via blood meal to BTV-2 and BTV-10.[24] While that study demonstrated *C. sonorensis* to be poorly competent for BTV-2, it would be constructive to further investigate if coinfections could overwhelm insect immunity and result in decreased lifespans. Likewise, the survival studies indicated that warmer temperatures correspond with a shorter mean survival time. This finding is also consistent with other studies across the literature.[24,52,53] In the context of climate change, the shorten lifespan of a vector may have induce a reciprocal tradeoff with increased early virogenesis.

While virogenesis was difficult to compare between temperature groups due to midge survivability differences, plaque assays consistently demonstrated that warmer temperatures

yielded earlier production of viral progeny. This finding is corroborated by previous BTV studies as well as in research of other arbovirus systems.[13,24,54,55] Rates of chemical reactions, which include viral nucleic acid synthesis and viral protein synthesis, increase with temperature.[16] This has been exemplified by measured rates of avian influenza virus hemagglutinin and neuraminidase synthesis and of enzymatic activity of RNA dependent RNA polymerase at different temperatures.[15] Furthermore, ambient temperature affects insect physiology and immune responses including expression of defensin and cecropin antimicrobial proteins and melanin.[56–58]

As virogenesis increases with temperature, we postulated that the frequency of reassortment would also increase with higher temperatures. In contrast, we found that the majority of sequenced plaques aligned with the BTV-17 parental genotype regardless of temperature. In light of this finding, it is important to iterate that while *C. sonorensis* demonstrated competency for both serotypes, single infection with BTV-17 had more pools of *C. sonorensis* with detectable BTV compared to single infection with BTV-10. Thus, BTV-17 may have optimal fitness over BTV-10 in *C. sonorensis* which resulted in the overwhelming representation of BTV-17 among the viral progeny. Similar to our findings, a coinfection study with BTV-1 and BTV-8 in cattle displayed predominant detection of BTV-8 even though cattle demonstrated equivalent susceptibility to both serotypes during single infections.[59,60] Explanations for this finding included biased detection of BTV-8 segment 2 via RT-qPCR (as sequencing was not performed in this study), viral interference, or increased BTV-8 tropism in the examined tissues.

The specific virus strains employed for the coinfections may also affect reassortment frequency. Successful reassortment requires that the parental strains coinfect the same cell,

package with each other's genomic segments, and have replicative compatibility. Superinfection exclusion, in which a viral infection already occupying a cell prevents a second infection from another virus, is a mechanism that inhibits cellular coinfection. The literature suggests that superinfection exclusion is less effective between viruses that are not as closely related.[61] However, requirements for segment packaging and replicative compatibility may favor reassortment between viruses that are more closely related.[62] Thus, it remains unclear whether genomic differences in parental strains may facilitate or hinder reassortment potential.

One pool of *C. sonorensis* earlier in the study at seven days post infection at 30°C and one pool of *C. sonorensis* later in the study at 19 days post infection at 20°C exhibited progeny virus with mixed genotypes (detection of both parental strains within one segment). The detection of mixed genotypes could be an artifact of plaque bleed over, in which two plaques may be close together and appear as one plaque, or a product of anomalous segment packing. Another explanation could be that BTV was found to egress from cells in lysosome-derived extracellular vesicles.[63] If extracellular vesicles contain both BTV-10 and BTV-17 progeny virus that infects a single cell this could confound the plaque isolation approach for identifying reassortant. As mixed plaques were found in earlier time points in the 30°C group as compared to the 20°C group, it would be compelling to investigate if reassortment frequencies could additionally be affected by duration of coinfection.

The findings of this study reflect the stringencies that reassortment requirements may have on reassortment as well as the knowledge gaps in our understanding of BTV replication cycle and host interactions. The overwhelming outcome of parental genotype representation in progeny plaques also highlights the balance between genetic stability and diversity needed for maintaining fitness in an alternating host cycle but having mechanisms for genetic alterations for

adaption in changing environments. As a parental serotype has demonstrated its capacity to survive the gauntlet of the alternating host cycle, any genetic changes may threaten its ability to replicate when transmitted to the next host. However, this must be balanced with evolution, where there is always the promise of increased fitness and potential viral emergence. In the context of climate change, these mechanisms require further interrogation, particularly in viruses of high consequence such as BTV.

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CHAPTER 3 – ASSESING REASSORTMENT BETWEEN BLUETONGUE VIRUS SEROTYPE 10 AND 17 AT DIFFERENT COINFECTION RATIOS IN *CULICOIDES SONORENSIS*

Introduction

Bluetongue virus (BTV) is the causative agent of bluetongue disease (BT) and has produced devastating epizootics in domestic and wild ruminant populations. Due to its serious ramifications on animal health globally, it is listed by the World Organization for Animal Health.[1,2] Transmitted to susceptible ruminant species by the *Culicoides* biting midge (Diptera: Ceratopogonidae), BTV is the prototype virus of the genus *Orbivirus* in the order *Reovirales*, family *Sedaroviridae.*[3,4] Viruses of order *Reovirales* are non-envelope and have double-stranded RNA with segmented genomes that are capable of reassortment. Specifically, BTV has ten genome segments with segment 2 encoding for the viral protein 2 (VP2) that confers the serological response in susceptible hosts. Conventional nomenclature distinguishes different BTV serotypes by the serological response incurred by VP2.[5,6] There are over 29 recognized BTV serotypes in circulation globally.[7] Currently, BTV serotypes in the United States are classified as “established” (3, 6, 10, 11, 12, 13, 17), “reported" (1, 2, 5, 9, 14, 15, 18, 19, 22, 24), or “not reported” (4, 7, 8, 16, 20, 21, 23, 25, 26).[8]

Though segment 2 directs conventional BTV nomenclature, the remaining nine segments encode proteins that are important for BTV infection and subsequent replication. Genomic segments 1, 3, and 4 encode VP1 (RNA-dependent RNA polymerase), VP3 (component of the inner capsid), and VP4 (a viral capping protein), respectively.[9–11] Segment 5 encodes for NS1 (which assists with viral translation and formation of microtubules) while segment 6 encodes for

VP5 (an outer shell protein that facilitates release of the viral core into the cytoplasm).[12–15] Segment 7 encodes for VP7, another inner shell protein.[10,11] NS2 aids in translation and formation of viral inclusion bodies and is encoded by segment 8. [12,16] The RNA helicase, VP6, is encoded by segment 9.[10] Leaky scanning of segment 9 also yields NS4, which may have interferon antagonism properties. [17,18] Finally, segment 10 encodes for NS3, NS3a, and NS5. NS3 and NS3a facilitate viral egress.[19,20] The newly recognized NS5 protein may have modulatory effects on the host cell response.[21]

Reassortment is an important mechanism for BTV evolution and allows for genomic segments to exchange between different parental serotypes, resulting in progeny with potentially novel phenotypes. However, reassortment requirements may limit the extent to which different parental BTV genotypes can exchange genetic material. In the field, it is necessary for the parental BTV genotypes to be circulating in the same geographical region at the same time (sympatric requirement) and they must be able to infect the same species of host (ecological requirement). Additionally, they must coinfect the same cell (coinfection requirement) and be able to package each other's genomic segments (segment packaging requirement) within a virion. The proteins encoded by the parental viruses must be able to function together to replicate more virus (replicative compatibility requirement). After those requirements have been met, the reassorted progeny virus must be able to compete with the parental virus (fitness requirement).[22]

Evidence of BTV reassortment is prevalent in experimental and field studies and is predicted to have effects on transmission, pathogenicity, and range of host diversity.[23–27] Reassortment has been observed in both the ruminant host and *Culicoides* vector, with past literature indicating that *Culicoides* is a more permissive host for reassortment of BTV using electrophoretic patterns.[23,24,28] However, there are few contemporary studies that have

investigated reassortment in the *Culicoides* using the more sensitive next-generation sequencing (NGS) platform.

Culicoides exposure to two different parental BTV serotypes can be from acquiring a blood meal from two different ruminant hosts each infected with a different serotype or from a single ruminant host that is infected with two different serotype. In either scenario, it is likely that the serotypes are not present in the blood at equal infectious titers. An early *in vitro* study investigated BTV reassortment in Vero cell culture infected with two different BTV serotypes at unequal multiplicity of infection (MOI) and found that the BTV serotype with the higher MOI contributed the majority of segments to the progeny virus.[29] Similarly, in this study we evaluate if different ratios of BTV serotype 10 (BTV-10) and BTV serotype 17 (BTV-17) fed to *Culicoides sonorensis*, the main vector of BTV in the United States, affect genotype and reassortment outcomes of progeny virus.[3] Additionally, we assess the rates of virogenesis to identify if the parental serotype virogenesis rates correspond to reassortment outcomes and to compare single infection to coinfection virogenesis. Identifying factors that affect reassortment will inform our understanding of BTV epidemiology and emergence of novel strains.

Materials and Methods

Viruses

BTV-10 California 1952 (BTV-10) (Bluetongue virus, type 10, strain 8, ATCC®VR-187™) and BTV-17 Colorado 2018 (BTV-17) (BTV serotype 17 CO 2018) were used to establish BTV single and coinfections in *Culicoides sonorensis*. The strain of BTV-10 was obtained from ATCC and was originally isolated from a sheep in California in 1952 and passaged eight times on BHK 21 cells.[30] The strain of BTV-17 was originally isolated from the whole blood of a sheep in Colorado in 2018 on CuVaW3 cells and subsequently passaged two

times on BHK 21 cells.[31] A third BTV strain was employed for a supplemental coinfection study, BTV- 17 California 1988 (BTV-17 CA) (BTV17 USA1988/CA). BTV-17 CA was isolated in California in 1988 from whole blood of clinically infected sheep and passaged six times on BHK 21 cells as described by DeMaula et al.[32] Whole genome sequences of each virus were determined previously by our lab and are available on GenBank (BTV-10 GenBank Accession MW456747-MW456756; BTV-17 GenBank Accession OQ798198-OQ798207, BTV-17 CA GenBank Accession MT952971-MT952980).[31,33,34] The specific strains of BTV-10 and BTV-17 were selected for this study because their relatively low nucleotide identities at the segment level facilitates distinguishability of genotypes by NGS (Table 3.1). BTV-17 CA was selected for a supplemental coinfection study with BTV-10 to investigate if BTV serotypes with higher nucleotide identities were more compatible for coinfection and subsequent reassortment (Appendix 2). Infectious titers for BTV-10, BTV-17, and BTV-17 CA were determined by 50% tissue culture infectious dose (TCID50) on BHK 21 cells as described previously and calculated by the Reed-Muench equation.[33,35]

Cells

BHK 21 cells were used to conduct plaque isolation and propagation on homogenized BTV infected *C. sonorensis.* BHK 21 cells were maintained in media containing Eagle's Minimum Essential Medium (EMEM) and supplemented with 10% heat-inactivated fetal bovine serum (FBS), 10% tryptose phosphate broth, and 1% penicillin-streptomycin (10,000 U/ml). Cells were incubated at 37°C with 5% CO_2 and passaged at approximately 90% confluency every three to four days.[33]

Table 3.1 – Nucleotide Pairwise Identity of BTV-10 and BTV-17 Genomic Segments

Segment	Protein encoded	% Pairwise Identity
Seg-1	VP1	96.1
Seg-2	VP2	68.8
Seg-3	VP3	97.3
Seg-4	VP4	96.3
Seg-5	NS1	96.9
Seg-6	VP5	79.5
Seg-7	VP7	95.8
Seg-8	NS2	96.2
Seg-9	VP6/NS4	96.9
Seg-10	NS3/NS3A/NS5	81.7

Culicoides sonorensis infection and maintenance

AK colony *C. sonorensis* (derived from wild *C. sonorensis* in Idaho 1973 and perpetuated by the USDA ARS, Manhattan, KS, USA) were acquired from USDA ARS.[36] Upon arrival, the *C. sonorensis* acclimatized for at least 24 hr at 25°C on a 12:12 hr light cycle. *Ad libitum* 10% sugar water was provided.[36] At time of infection via virus-spiked blood meal, the *C. sonorensis* were three to four days post-emergence. Mechanically defibrinated sheep blood (Hemostat Laboratories, Dixon, CA, USA or Lampire Biological Laboratories, Everett, PA, USA) was evaluated for presence of BTV virus and antibodies via pan BTV qRT-PCR and cELISA (VMRD, Pullman, WA, USA). The infection experiment was divided into two studies, Study A and Study B, due to limited numbers of *C. sonorensis.* Study A included *C. sonorenesis* infection groups fed negative, BTV-10, BTV-17, or combined BTV-10:BTV17 at ratios of 90:10, 75:25, or 50:50 spiked blood meals. Study B included *C. sonorenesis* infection groups fed negative, BTV-10, BTV-17, or combined BTV-10:BTV17 at ratios of 50:50, 25:75, or 10:90 spiked blood meals. BTV was added to the blood to produce a total TCID50/ML of 1 x 10^5 for each infection group within study A and study B (Table 3.2). A third supplemental study, study

C included *C. sonorenesis* infection groups fed negative, BTV-10, BTV-17 CA, or combined BTV-10:BTV17 CA at a ratio of 50:50 spiked blood meals. For negative control blood, media used to suspend virus was applied at equal volume as the infectious blood feeding groups. Blood was provided to *C. sonorensis* in glass bell feeders that maintained blood at 37°C through parafilm membranes. Blood feeding occurred for 80 min.

Table 3.2 – BTV $TCID_{50}$ /mL for Infection Groups

Study A Infection Group	**BTV-10 $TCID_{50}$/mL**	**BTV-17 $TCID_{50}$/mL**	**Total $TCID_{50}$/mL**
Negative	N/A	N/A	N/A
BTV-10	1×10^5	N/A	1×10^5
BTV-17	N/A	1×10^5	1×10^5
90:10 BTV-10:BTV-17	9×10^4	1×10^4	1×10^5
75:25 BTV-10:BTV-17	7.5×10^4	2.5×10^4	2×10^5
50:50 BTV-10:BTV-17	5×10^4	5×10^4	1×10^5
Study B Infection Group	**BTV-10 $TCID_{50}$/mL**	**BTV-17 $TCID_{50}$/mL**	**Total $TCID_{50}$/mL**
Negative	N/A	N/A	N/A
BTV-10	1×10^5	N/A	1×10^5
BTV-17	N/A	1×10^5	1×10^5
50:50 BTV-10:BTV-17	5×10^4	5×10^4	1×10^5
75:25 BTV-10:BTV-17	2.5×10^4	7.5×10^4	2×10^5
90:10 BTV-10:BTV-17	1×10^4	9×10^4	1×10^5

After blood feeding, *C. sonorensis* were immobilized by placement into a -20°freezer for five minutes. Blood fed *C. sonorensis* were then sorted on a modified chill table and placed in groups of approximately 100 *C. sonorensis* per container according to BTV infection group. The same BTV stocks were used in Study A and Study B. Immediately following sorting, five to ten blood-fed midges were collected from each infection group and screened for ingestion of virus by pan BTV qRT-PcR to establish that the *C. sonorensis* were exposed to virus at the blood feeding.

For the remainder of the study, *C. sonorensis* were housed in non-treated paper tube containers (Rigid Paper Tube Corporation, Wayne, NJ, USA) with sheer panty hose stretched over the lid for air exchange and feeding. Sugar water (10% w/v) was available at all times with a cotton wick on each container. *C. sonorensis* were maintained at 25°C ± 2°C with a 12:12 hr light cycle.

C. sonorensis collections

To longitudinally evaluate virogenesis via qRT-PCR, five *C. sonorensis* were collected in triplicate from each treatment group every other day through day ten post infection or until no specimens remained. At day ten post infection, according to availability, ten *C. sonorensis* from each treatment group was collected in triplicate for plaque assays. *C. sonorensis* collected were stored at -80°C until downstream processing. Prior to nucleic acid extraction or plaque assays, *C. sonorensis* were manually homogenized with a sterile pestle in EMEM volume of 50 µL per *C. sonorensis* (i.e., 250 µL for pools of five *C. sonorensis* and 500 µL for pools of ten *C. sonorensis*).[37]

Single and coinfection plaque assays

Plaque assays were performed on the homogenized pools of ten *C.* sonorensis from each infection group. Homogenized pools were vortexed and briefly centrifuged. 400 µL of the supernatant was syringe sterile-filtered (0.22 µM Millex-GV syringe filter, MilliporeSigma, Burlington, MA, USA) and further diluted in EMEM at 1:2, 1:10, 1:100, 1:1000, and 1:10,000 dilutions.

48 hours prior to commencing plaque assays, BHK 21 cells were seeded in 6-well plates at 1.0 x 10^5 cells/well and maintained as described above. Immediately before inoculations with *C. sonorensis* homogenate dilutions, confluent monolayers were washed one time with 500 µL

PBS pH 7.5 per well. Each well was inoculated with 500 µL of one of the *C. sonorenesis* dilutions and incubated for 1 hr at 37°C with rocking every 15 minutes to distribute the virus. Inoculum was then aspirated off the cells and the cells were washed with PBS pH 7.5. This was followed by overlay with 2 mL of 3:1 BHK 21 media: 2% agarose in Earle's Buffered Salt Solution (EBSS). The 6-well plates were incubated at 37°C until plaques were evident by microscopy or for 96 hours followed by a 1 mL overall of 3:1 BHK media: 2% agarose in EBSS with 0.1% neutral red stain. Individual plaques were collected 8-24 hours after the second overlay when plaques were visibly evident. Agarose plugs collected from discretely isolated plaques were propagated in individual wells of 48-well plates with BHK 21 cells (4.65×10^4 per well). The supernatants of the propagated plaques were collected when cytopathic effect was prominent and stored at -80°C until further processing.[37]

Nucleic acid extraction and DNase treatment

Nucleic acid was extracted from supernatants of homogenized *C. sonorensis* pools and supernatants of propagated plaque using the MagMAX Pathogen RNA/DNA kit from Applied Biosystem (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions for low cell content samples. However, the pools of *C. sonorensis* were processed on the KingFisher Flex robot (Thermo Fisher, Waltham, MA) and eluted in 90 µl and the plaques were processed manually and eluted in 45 µl to increase nucleic acid concentration for downstream sequencing. The *C. sonorensis* that were collected in pools of five to assess virogensis rates via qRT-PCR were DNAsed with DNase 1, RNase free (Thermo Fisher) after homogenization and extraction as described by Kopanke et al.[37] The extracted homogenized *C. sonorensis* collected in pools of ten and their subsequent isolated propagated plaques were treated with TURBO DNA-*free*™ kit (Invitrogen) and incubated with 7.5 M LiCl to respectively deplete DNA and precipitate single

stranded RNA to enrich for dsRNA as described by Kopanke et al.[37] Afterwards, samples were treated with a 1.25 x MagMAX Pathogen RNA/DNA kit clean-up step to remove excess salts.

Pan BTV and cox1 qRT-PCR assay

Extracted RNA from *C. sonorensis* homogenates were screened for BTV detection in duplicate using a universal pan BTV qRT-PCR that recognize BTV segment 10 as previously described using SuperScript III One-Step qRT-PCR reagents (Themo Fisher) at half-reaction volumes.[37,38]

Normalization for variations in extraction efficiency was accomplished by performing, in duplicate, a qRT-PCR based on *Culicoides* mitochondrial cytochrome c oxidase subunit 1 (*cox1*) as described by Kopanke et al using a FAM-based probe (3'FAM-TGAATACTT/ZEN/CCTCCTTCTCTTTCTT - 3IABkFQ/5', Integrated DNA Technologies, Coralville, IA).[37] Positive and negative controls for BTV and *Culicoides cox*1 were run with each plate. Additionally, to confirm adequate DNAse treatment, a no-reverse transcriptase (no-RT) control was performed. The ΔCt method based on mean Ct values for BTV and *cox*1 for each sample ($Ct_{BTV} - Ct_{cox1} = \Delta Ct_{normalized}$) was applied to determine BTV Ct normalized values by *Culicoides cox1* Ct values.

Additionally, BTV-10 and BTV-17 serotype specific qRT-PCR, as described by Maan et al., was performed on pools of *C. sonorensis* used for plaque propagation to characterize if there was presence of both BTV-10 and BTV-17 segment. Ct values less than 36 were considered positive for detection.[39]

Library preparation and whole genome sequencing

Shotgun whole genome sequencing was performed on the extracted, DNAsed and LiCl treated homogenized *C. sonorensis* collected in pools of ten and their subsequent isolated

propagated plaques to determine pool and plaque genotypes. Negative water controls as well as positive controls BTV-10, BTV-17, and BTV-10:BTV-17 at 50:50 were performed with each library prep and sequencing run. Additionally, positive controls of BTV-17 CA and BTV-10: BTV-17 CA at 50:50 were performed. Sample libraries were prepared using the NEBNext® Ultra II Directonal RNA Library Prep Kit of for Illumina® (New England BioLabs Inc., Ipswich, MA, USA), according to the Section 4 Protocols for use with Purified mRNA or rRNA depleted RNA with the following CDC modifications for double-stranded RNA: 1) RNA fragmentation was performed at 90°C for 1 min and 2) first strand cDNA synthesis reaction was 10 min at 25°C, 30 min at 42°C, 15 min at 70°C, hold at 4°C. NEBNext Multiplex Oligos for Illumina (96 Index Primers) were applied for sample identification according to manufacturer's protocol. Libraries were assessed for DNA quality and concentration with Qubit high sensitivity DNA reagents on the Qubit 2.0 fluorometer (Thermo Fisher, Waltham, MA, USA) and High Sensitivity D1000 DNA screentape on the TapeStation 2200 or 4150 instrument.

Approximately 60 samples were pooled at a time and subsequently size-selected for inserts from 300-900 base pairs with base pair size fractionation on a 1.5% agarose gel. After the appropriate region was excised from the gel, the sample was reextracted using the QIAquick Gel Extraction Kit according to the manufacturer's protocol (Qiagen, Hilden, Germany) followed by a bead-clean up using NEBNext® Ultra II Directonal RNA Library Prep Kit of for Illumina® Section 4.9 protocol. The pool was analyzed with the Qubit fluorometer and TapeStation instruments as described above and then quantified with the KAPA Library Quantification kit (KAPA Biosystems) according to kit instructions. The NextSeq 500/550 Mid Output Kit v2.5 (300 cycles) was used to perform paired-end sequencing (1 x 150) on a NextSeq 500 sequencer instrument (Illumina, USA).

BTV analysis pipeline and bioinformatics

Libraries were demultiplexed and then analyzed using the stenglein-lab/ btv_segment_table pipeline (https://github.com/stenglein-lab/btv_segment_table) that uses codes and infrastructure developed by the n-f core community.[40,41] This pipeline was designed to quantify the number of reads in a sample that map to each of the ten bluetongue virus genomic segments in order to determine the parental strain origin for each segment for assessment of reassortment. Pre-processing in this pipeline includes trimming of low quality sequences, bases, and adapter sequences using Cutadapt with Cd-hit-est to collapse duplicate read pairs. Reads were aligned in Bowtie 2 to the reference sequences (BTV-10 and BTV-17) that we provided. Default parameters were applied with the exception of the BTV Reference Sequences. FASTA files of BTV-10 and BTV-17 reference sequences used in this study were formatted and employed with the --refseq_fasta command line option.

Statistical Analysis

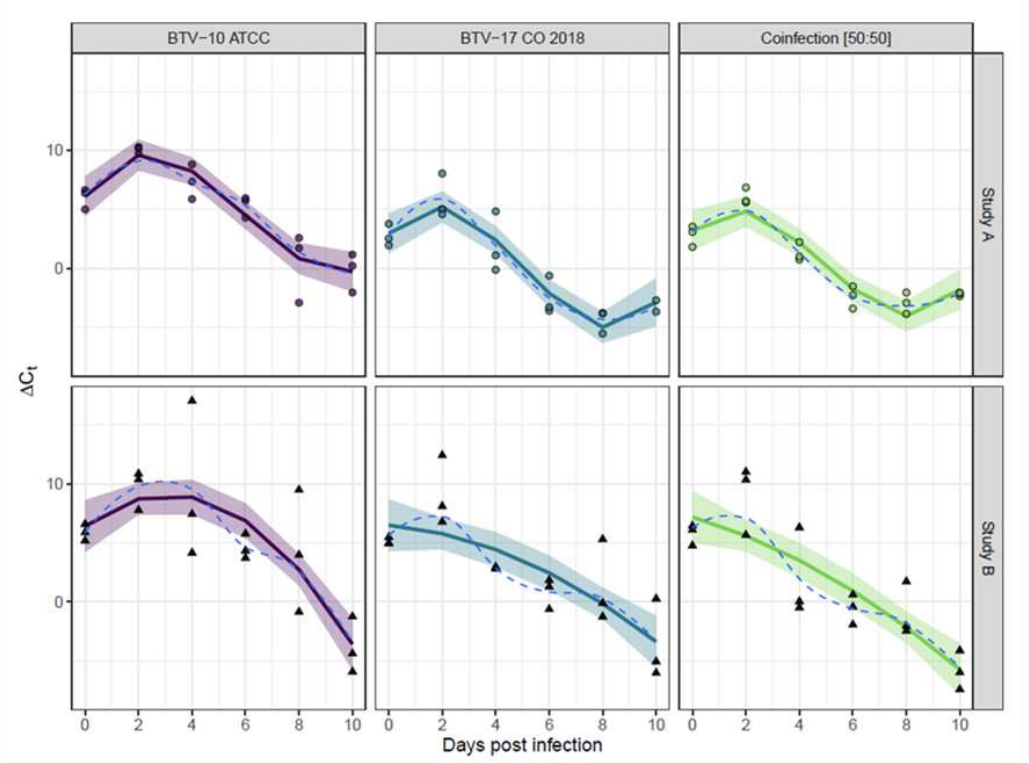
Rates of virogenesis comparisons of BTV-10 single infection, BTV-17 single infection and BTV-10: BTV-17 50:50 between Study A (fit with an interaction of group to a 3rd degree polynomial) and Study B (fit with an interaction of group to a 2nd degree polynomial) were performed on the linear portions of the curves by pairwise comparison with calculations for standard error, z ratio statistic, and P-value (adjusted with Tukey's HSD method).[42] For ΔCt at day zero *post-hoc* full pairwise comparison, the conservative Scheffe adjustment to p-values was employed.[43] Similar comparisons were made between infection groups within a study and in the supplemental study C. Statistics were performed in open source software R version 4.2.1 (2022-06-23) with linear trends calculated with emmeans package 1.7.5.[44] Bar graphs and heat maps of plaque genotypes were formulated with GraphPad Prism 8.1.0.

Results

BTV-10 and BTV-17 demonstrate similar rates of virogenesis in C. sonorensis

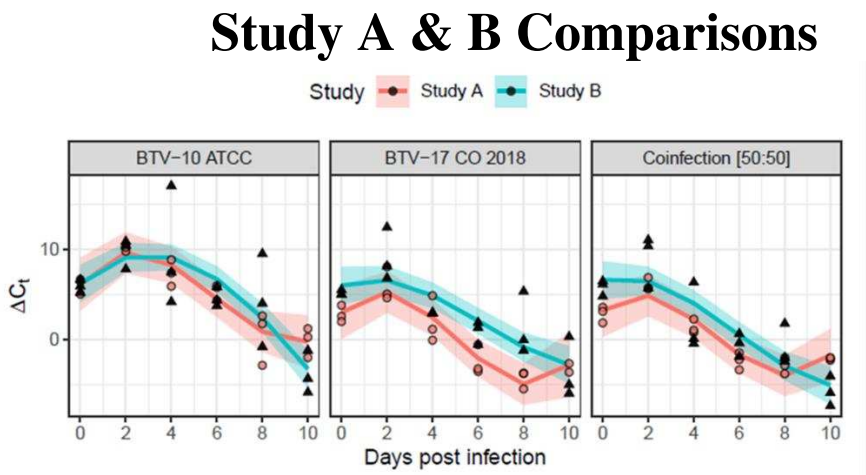
First, it was necessary to establish that colony *C. sonorensis* can support replication of both BTV-10 and BTV-17 and that the serotypes have similar rates of virogenesis. This was to assess if one serotype would overtly dominate infection as demonstrated in earlier studies. To evaluate rates of virogenesis between BTV-10 and BTV-17, *C. sonorensis* were provided blood meals with either BTV-10 or BTV-17 spiked at equal titers. The presence of BTV was assessed via Ct values of pan-BTV qRT-PCR performed on pools of five *C. sonorensis* collected in triplicated every other day and normalized by Ct values of the housekeeping gene *cox1*. The resulting $\Delta Ct_{normalized}$ was plotted over time and fit to a curve. Pairwise comparison between the linear portions of the curve demonstrated no significant differences, indicating that the presence of BTV RNA was similar between single infections of BTV-10 and BTV-17, as well as BTV-10 and -17 in 50:50 ratio infection groups. This was performed for both study A and study B (Figure 3.1). Furthermore, the single infection and 50:50 coinfection group curves were compared between study A and study B to ensure consistency between studies. Pairwise comparison between study A and study B curves demonstrated no significant differences (Figure 3.2). A similar curve was made for supplemental study C (BTV-10 and BTV-17 CA coinfection) and no significant differences were found between treatment groups (Appendix 3).

BTV-10 & -17 Comparisons



Contrast	Difference	SE	z ratio	p-value
Study A				
BTV 10 ATCC - BTV 17 CO	0.43	0.41	1.07	0.54
BTV 10 ATCC - BTV 10&17 CO 50:50	0.14	0.41	0.34	0.94
BTV 17 CO - BTV 10&17 CO 50:50	-0.30	0.41	-0.73	0.75
Study B				
BTV 10 ATCC - BTV 17 CO	-0.01	0.21	-0.07	1.00
BTV 10 ATCC - BTV 10&17 CO 50:50	0.29	0.21	1.40	0.35
BTV 17 CO - BTV 10&17 CO 50:50	0.30	0.21	1.47	0.31

Figure 3.1 - BTV-10, BTV-17, and BTV-10:BTV-17 50:50 Infection Groups Demonstrate Similar Virogenesis Rates in *C. sonorensis*. BTV-10 (in purple), BTV-17 (in blue), and coinfection of BTV-10: BTV-17 50:50 (in green) infection groups demonstrated no statistical difference as determined by pairwise comparison of the linear portion of curve. Each point (dot for study A and triangle for study B) represents the ΔCt of pool of *Culicoides* (n=5) collected at the indicated day post infection. The ΔCt is calculated as the difference between mean pan BTV CT and *cox1* Ct. Study A is fit with an interaction of group to a 3rd degree polynomial and Study B is fit with an interaction of group to a 2nd degree polynomial. The solid line indicates the model curve with a 95% confidence band. Standard error, z ratio statistic, and p-value adjusted with Tukey's method are given for each comparison.



Study A - Study B trend difference

Treatment	Difference	SE	t-ratio	p-value
BTV 10 ATCC	-0.71	0.62	-1.14	0.26
BTV 17 CO	-0.90	0.63	-1.44	0.15
BTV 10&17 CO 50:50	-0.24	0.62	-0.38	0.71

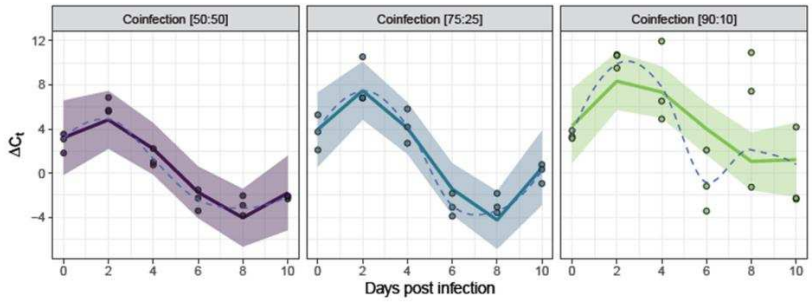
Figure 3.2 - Infection Groups Between Study A and B Demonstrate Similar Virogenesis Rates in *C. sonorensis*. BTV-10 (left), BTV-17 (center), and coinfection of BTV-10: BTV-17 50:50 (right) infection groups where compared between study A and B and demonstrated no statistical difference as determined by pairwise comparison of the linear portion of curve. Each point (dot for study A and triangle for study B) represents the ΔCt of pool of *Culicoides* (n=5) collected at the indicated day post infection. The ΔCt is calculated as the difference between mean pan BTV CT and *cox1* Ct. Study A and B are fit with an interaction of group to a 3rd degree polynomial. The solid line indicates the model curve with a 95% confidence band. Standard error, t ratio statistic, and p-value adjusted with Tukey's method are given for each comparison.

BTV-10 and BTV-17 coinfections at different ratios demonstrate similar rates of virogenesis in C. sonorensis

Similar to the single infection groups, $\Delta Ct_{normalized}$ was assessed over time for each of the different ratio infection groups. The rationale for this assessment was to evaluate if coinfection at different ratios had a synergistic effect on virogenesis or remained stable across treatment groups. If a treatment group had increased virogenesis, it would be interesting to see if that group had corresponding increased frequency of reassortment. However, pairwise comparisons

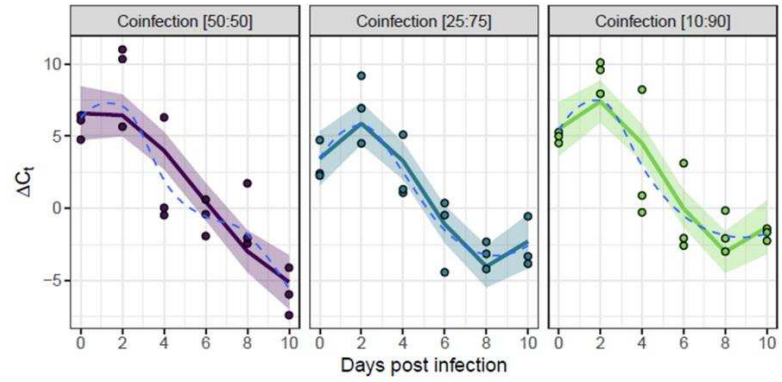
between the linear portions of the curves of each treatment group within study A or B determined there were no significant differences. This supported that the virogenesis of the combined titer of the two serotypes performed similarly across different ratio treatment groups (Figure 3.3).

Study A Coinfection Comparisons



Contrast	Difference	SE	z ratio	p-value
BTV 10&17 CO 50:50 - BTV 10&17 CO75:25	0.83	0.81	1.02	0.57
BTV 10&17 CO 50:50 - BTV 10&17 CO 90:10	-0.31	0.81	-0.38	0.92
BTV 10&17 CO75:25 - BTV 10&17 CO 90:10	-1.13	0.81	-1.40	0.35

Study B Coinfection Comparisons



Contrast	Difference	SE	z ratio	p-value
BTV 10&17 CO 50:50 - BTV 10&17 CO 25:75	0.46	0.46	1.02	0.57
BTV 10&17 CO 50:50 - BTV 10&17 CO 10:90	0.54	0.46	1.17	0.47
BTV 10&17 CO 25:75 - BTV 10&17 CO 10:90	0.07	0.46	0.16	0.99

Figure 3.3 – Infection Groups at Different Ratios Demonstrate Similar Virogenesis Rates in *C. sonorensis*. Study A) BTV-10: BTV-17 infection groups are represented by 50:50 (in purple), 75:25 (in blue), and 90:10 (in green) Study B) BTV-10: BTV-17 infection groups are represented by 50:50 (in purple), 27:75 (in blue), and 10:90 (in green). Infection groups have similar virogenesis rates within studies and between studies A and B. Each point represents the ΔCt of pool of *Culicoides* (n=5) collected at the indicated day post infection. The ΔCt is calculated as

the difference between mean pan BTV CT and *cox1* Ct. Study A and B are fit with an interaction of group to a 3rd degree polynomial. The solid line indicates the model curve with a 95% confidence band. The dashed line represents the mean of the data. Infection groups demonstrated no statistical difference as determined by pairwise comparison of the linear portion of curve. Standard error, z ratio statistic, and p-value adjusted with Tukey's method are given for each comparison.

BTV-10 and BTV-17 serotype detection in pools of C. sonorensis

To prioritize the plaques propagated from coinfected *C. sonorensis* for sequencing, the *C. sonorensis* pools used for plaque assays were evaluated for the presence of both BTV-10 and BTV-17 via BTV-10 and BTV-17 serotype-specific qRT-PCR. Up to ten plaques were sequenced from pools that had both serotypes represented by qRT-PCR and up to five plaques were sequenced from pools that only had one serotype represented by qRT-PCR (if there were adequate number of plaques isolated). Notably, all pools, with the exception of pools B and C from the 25:75 coinfection group, and all three pools from the 10:90 coinfection group, had both serotypes represented by qRT-PCR (Table 3.3). Sequencing of additional pools was attempted; however, due to low reads, most of the pools could not be adequately characterized.

Reassortant progeny detected in plaques propagated from coinfected C. sonorensis

Progeny plaques isolated from the plaque assays were classified as either BTV-10 or BTV-17, a reassortant genotype, or a mixed genotype as determined by sequencing results. If 90% or more of the reads were aligned to one parental serotype segment, and all ten segments aligned with the same parental serotype, the plaque was classified as having the genotype of that parental serotype (BTV-10 or BTV-17). If 90% or more of the reads were aligned to one parental serotype segment, but not all segments came from the same parental strain, the plaque was classified as reassortant. Mixed genotypes were plaques that contained at least one segment that aligned to both parental serotypes (less than 90% of the reads aligned with one parental

serotype). Of the 121 plaques sequenced across all infection groups in this study, only nine were reassortant and ten were mixed, with the rest having identical genotypes to BTV-10 or BTV-17. Study A coinfection group 75:25, pool A, is particularly compelling as it produced five of the reassortant plaques, four of the mixed plaques, and only contained one BTV-10 plaque. In study A, the 50:50 coinfection group's pools B and C each produced one reassortant, and in study B, the 50:50 coinfections group's pool B and the 25:75 coinfection group's pool A each produced one reassortant (Table 3.3). For supplemental study C, which evaluated coinfection of BTV-10 with BTV-17 CA, it is difficult to assess plaque genotypes with any confidence. For sequencing controls, see Appendix-1.

Table 3.3 – Serotype & Genotype Classifications of Pools & Plaques from *C. sonorenesis* Infection Groups. Serotype of pools were determined by serotype specific qRT-PCR. (+) indicates a Ct value of 36 or lower. Plaque genotypes were determined by NGS.

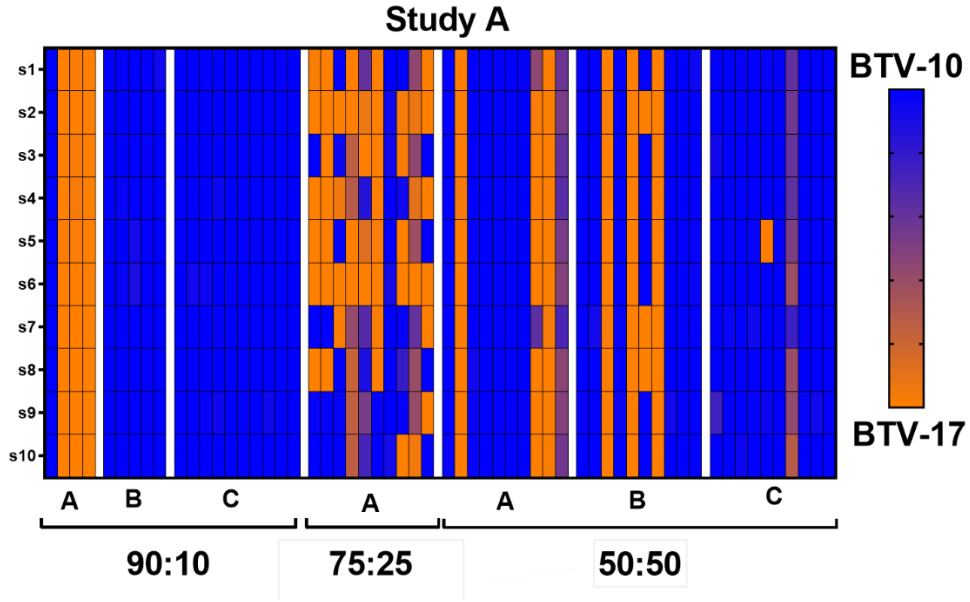
						Genotype							
			PCR Serotype Detection			BTV-10		BTV-17		Reassortant		Mixed	
	Infection Group	Pool	BTV-10	BTV-17	No. Plaques Sequenced	No.	%	No.	%	No.	%	No.	%
Study A	**90:10**	A	+	+	4	1	25	3	75	0	0	0	0
	90:10	B	+	+	5	5	100	0	0	0	0	0	0
	90:10	C	+	+	10	10	100	0	0	0	0	0	0
	90:10 Total				19	16	84	3	16	0	0	0	0
	75:25	A	+	+	10	1	10	0	0	5	50	4	40
	50:50	A	+	+	10	6	60	2	20	0	0	2	20
	50:50	B	+	+	10	6	60	3	30	1	10	0	0
	50:50	C	+	+	10	7	70	0	0	1	10	2	20
	50:50 Total				30	19	63	5	17	2	7	4	13
Study B	**50:50**	A	+	+	10	10	100	0	0	0	0	0	0
	50:50	B	+	+	10	4	40	5	50	1	10	0	0
	50:50	C	+	+	10	10	100	0	0	0	0	0	0
	50:50 Total				30	24	80	5	17	1	3	0	0
	25:75	A	+	+	10	7	70	0	0	1	10	2	20
	25:75	B	-	+	5	0	0	5	100	0	0	0	0
	25:75	C	-	+	5	0	0	5	100	0	0	0	0
	25:75 Total				20	7	35	10	50	1	5	2	10
	10:90	A	-	+	5	0	0	5	100	0	0	0	0
	10:90	B	-	+	5	0	0	5	100	0	0	0	0
	10:90	C	-	+	2	0	0	2	100	0	0	0	0
	10:90 Total				12	0	0	12	100	0	0	0	0

Reassortants may have preferential parental segment combinations.

Coinfection group 75:25 from study A is particularly distinctive as the majority of plaques are either reassortants or mixed genotypes (Table 3.4 and Figure 3.4 A). Although statistical assessment is not feasible due to low sample number, it is notable that all reassortant plaques in this pool have segment 2, 4, and 6 aligned with BTV-17 while four of five have segments 9 and 10 aligned with BTV-10. Segment 9 and 10 also align with BTV-10 in most of the remainder of the reassortant plaques with a total of 7 and 8 reassortants respectively. Additionally, all nine reassortant plaques are unique, with none displaying the same combination of genotypes (Table 3.4 and Figure 3.4).

Table 3.4 – Segregation Ratios (BTV-10/BTV-17) of Genome Segments from Pools with Reassortant Genotypes.

Study	Infection Group Pool	seg-1	seg-2	seg-3	seg-4	seg-5	seg-6	seg-7	seg-8	seg-9	seg-10	Total ratio by infection group
A	75:50 Pool A	1/4	0/5	3/2	0/5	2/3	0/5	2/3	2/3	4/1	5/0	19/31
	50:50 Pool B	1/0	0/1	1/0	1/0	1/0	1/0	0/1	0/1	1/0	1/0	7/3
	50:50 Pool C	1/0	1/0	1/0	1/0	0/1	1/0	1/0	1/0	1/0	1/0	9/1
B	50:50 Pool B	1/0	0/1	0/1	1/0	1/0	0/1	1/0	0/1	1/0	1/0	6/4
	25:75 Pool A	0/1	1/0	0/1	0/1	1/0	1/0	1/0	1/0	0/1	0/1	5/5
Total ratio by seg		4/5	2/7	5/4	3/6	5/4	3/6	5/4	4/5	7/2	8/1	46/44



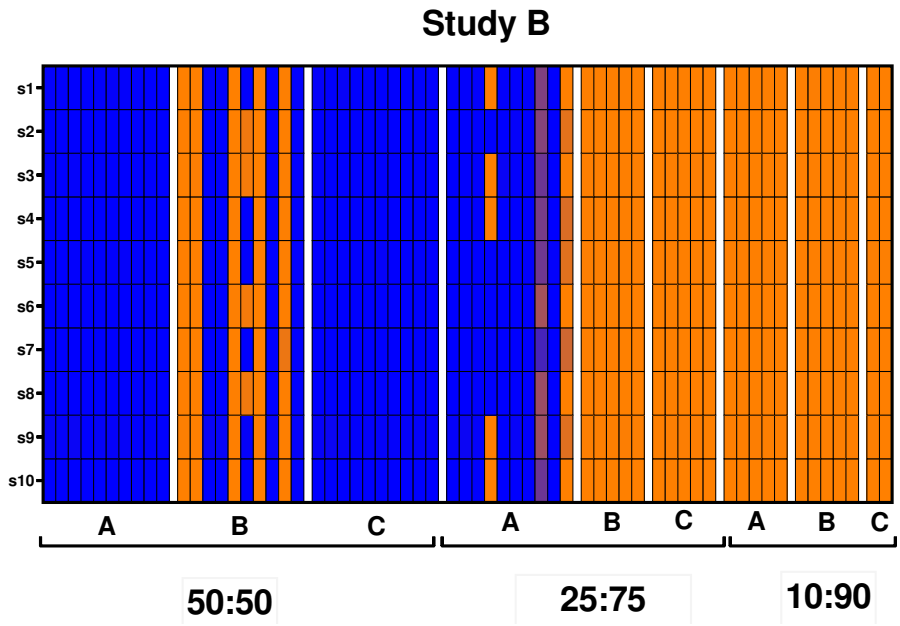


Figure 3.4. – Reassortant Progeny Plaques Present in 75:25, 50:50, and 25:75 Infection Groups. Plaque genotypes that were sequenced from study A and B are depicted by heat maps. Each column on the x -axis represents individual plaques with pools separated by white margins and indicated by letters A, B, and C. The y -axis represents the ten different genomic segments of BTV. Blue indicates that 100% of the sequencing reads for that segment aligns with BTV-10. Orange indicates that 100% of the sequencing reads for that segments align with BTV-17. Color gradient in between blue and orange indicate that both parental strains aligned with the segment.

There is modest correspondence between coinfection ratios and plaque genotypes outcomes

Overall, a trend was observed in which the parental strain with the higher titer at the time of blood feeding corresponded to a higher prevalence of segments detected in progeny virus. This is demonstrated with the plaques from the 90:10 coinfection group in study A, mostly consisting of BTV-10 segments, and the 25:75 and 10:90 coinfection groups in study B, mostly consisting of BTV-17 segments (Figure 3.5). However, there are notable exceptions to this trend, particularly between pools within infection groups and in the 75:25 coinfection group.

In study A, segments from plaques propagated from pools B and C of *C. sonorensis* from the 90:10 coinfection group exclusively aligned with BTV-10, the higher titer parental serotype. However, segments from plaques in pool A of the 90:10 coinfection group aligned primarily with BTV-17 (75%). All plaques in this coinfection group aligned with a parental serotype with no mixed segments (a segment with reads from both parental serotype) or reassortants detected (Table 3.4, Figure 3.5). In the 75:25 coinfection group, reassortment and mixed genotypes were abundant and did not correspond with the trend of the higher contribution of segments from the parental serotype with the higher titer. In this case, 46% of the segment reads aligned with BTV-17, the lower titer parental serotype, with only 33% percent of the segments aligned with BTV-10. The remaining 21% of the segments had alignments to both BTV-10 and BTV-17. Unfortunately, only one pool of *C. sonorensis* was available for plaque isolation in the 75:25 coinfection group due to decreased midge survivorship. In the study A 50:50 coinfection group,

segments from both BTV-10 and BTV-17 were represented in each pool. Pool A had 60% BTV-10, 28% BTV-17, and 12% mixed segments. Pool B had 67% BTV-10, 33% BTV17, and no mixed segments. Pool C had 88% BTV-10, 1% BTV-17, and 11% mixed segments (Figure 3.5).

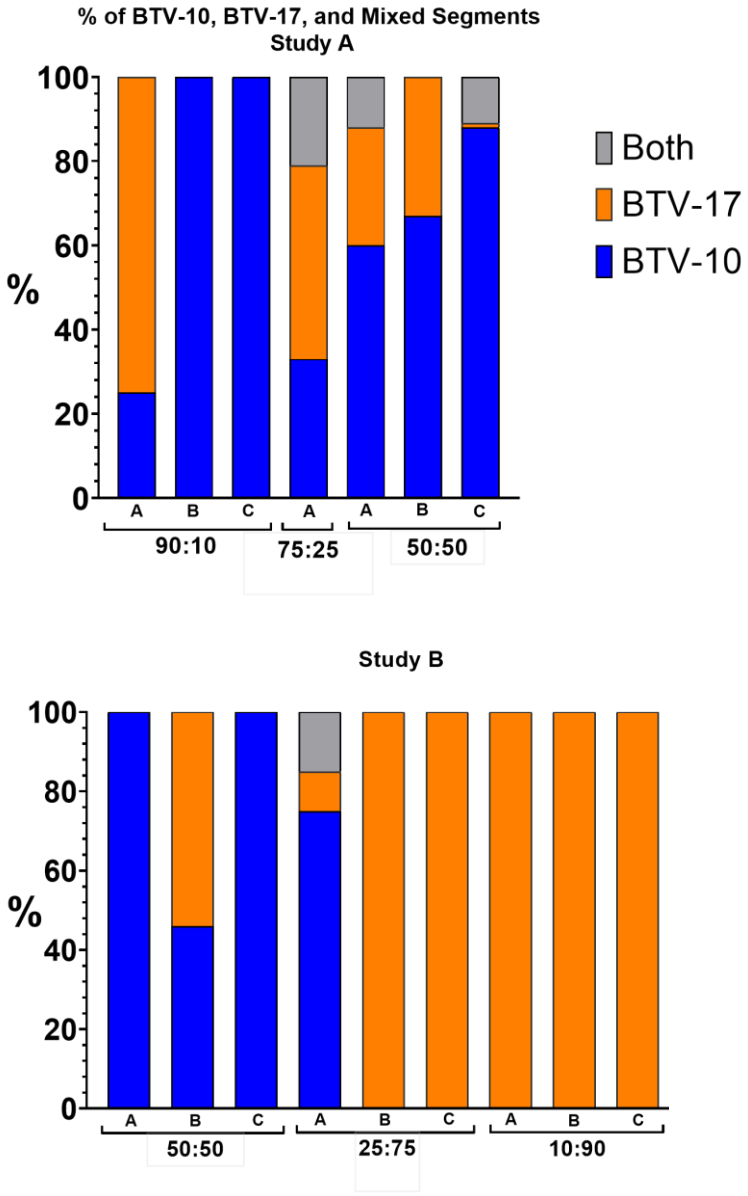


Figure 3.5 – Percentage of Segments that Align with BTV-10 or BTV-17 Demonstrates Modest Correspondence to Infection Group Coinfection Ratio. The y -axis indicates the percentage of segments that align with a parental strain out of total segments within that pool. The columns represent each pool within and infection group. Blue represents BTV-10. Orange represents BTV-17. Gray represents segments with both BTV-10 and BTV-17 sequences present.

In study B there was presence of BTV-10 segments in all of the 50:50 pools (100% in pools A and C and 46% in pool B) and in one of the 25:75 pools (75% in pool A). The remaining segments of pool A in the 25:75 coinfection group consisted of 10% BTV-17 and 15% mixed segments. The remainder of the pools in the 25:75 and 10:90 coinfection groups consisted exclusively of BTV-17 segments (Figure 3.5).

Discussion

Reassortment is an important mechanism for generating large genetic changes in BTV. Understanding the determinants that support or inhibit reassortment will facilitate our understanding of BTV's evolutionary trajectory. In this study we investigated how different coinfection ratios of parental serotypes affect progeny virus outcomes in the *Culicoides* BTV vector. A supplemental study investigating confection of BTV-10 and BTV-17 CA was also performed to investigate reassortment outcomes between two serotype strains with high pairwise identity. Unfortunately, the high pairwise identity between BTV-10 and BTV-17 CA inhibited our analysis of plaque genotypes.

Establishing that virogenesis rates of the two different serotypes BTV-10 and BTV-17 employed in this study were similar in the colony *C. sonorensis* gave insight as to whether there was potential for coinfection and subsequent reassortment. If the *C. sonorensis* were not competent for one of the serotypes or if the virogenesis rates were drastically different, the dominant serotype may prevent a coinfection from occurring regardless of titer ratio. Previous studies have demonstrated the varying competencies *Culicoides* species and even between specific *C. sonorensis* colonies have for different BTV serotypes.[37,45] Comparison of virogenesis rates between the different coinfection ratios was also performed to investigate if coinfection could have any synergistic effect on viral replication in the *Culicoides*. This could be due to

altered immune function, dissemination, or perhaps a more fit reassortant progeny. However, across all infection groups, viral kinetics were consistent.

Reassortment was detected in this study, however the occurrence of reassortment was not as robust as reported in previous *in vitro* or *in vivo* studies.[23–25,28,29,33] In the combined study A and B 50:50 infection groups only three progeny were reassortant and four were mixed genotypes out of the 60 plaques evaluated. Ramig et al. *in vitro* studies applying different BTV-10 and BTV-17 strains at equal MOIs resulted in 54% of the progeny analyzed being reasortant.[29] In Samal et al. *in vivo* studies with *C. sonorensis* reassortants ranged from 7% to 78%.[23] Interestingly, Ramig et al. observed that there was quite extensive experimental variation of reassortment frequencies and ratio segregations in their *in vitro* infection studies and among other BTV *in vivo* studies. It was surmised that this was due to challenges and errors in reproducing accurate titers between studies and amplification of progeny genotypes formed early in infection.[23,24,28,29]

The underwhelming prevalence of reassortants in our study could be related to the specific serotypes/strains employed. Although the colony *C. sonorensis* could support replication of both BTV serotypes there may have been limitations to the extent successful reassortment could occur. This could be due to superinfection exclusion phenomenon, weak segment packaging signals, or incongruent replicative compatibility. Current thought is that viruses that are not as closely related may be able to overcome superinfection exclusion easier than viruses that are closely related.[46] In contrast, replicative compatibility would suggest that the need for compatible virus components would favor reassortment between viruses that are more closely related.[47] This is was the reason for the supplementary coinfection study with BTV-17 CA. As BTV-10 with BTV-17 has lower pairwise identity and BTV-10 with BTV-17 CA has higher

pairwise identity, we wanted to investigate if genetic similarities would permit more reassortant events. However, the caveat is the higher the pairwise identity, the more difficult it is to distinguish genotypes. Another requirement for reassortant progeny success is that they must be able to compete with the parental serotypes. Perhaps BTV-10 and BTV-17 have elevated fitness compared to most reassorted progeny outcomes, effectively making it difficult for reassortants to persist.

However, there was one distinct group of plaques that mostly comprised of reassortant and mixed plaques. The abundance of reassortant progeny in infection group 75:25 compared to other infection groups discloses two questions: 1) why does 75:25 have more reassortants and mixed plaques than the other treatment groups?; and 2) although it has a higher BTV-10 ratio, why do more progeny segments align with BTV-17? It could be that 75:25 was the most optimal ratio for parental fitness equivalence or perhaps the ratio simulated asynchronous infection optimal for superinfection. However, the 90:10 infection group and the 50:50 infection group both having more representation of BTV-10 in its progeny segments which is inconsistent with those explanations. Unfortunately, we only had one replicate of pooled *C. sonorensis* in the 75:25 group due to poor survivability, making interpretations of this outcome a further challenge. Another potential explanation is that progeny with segments 2, 4, 6, from BTV-17 and segments 9, 10 from BTV-10 have fitness gains over the parental serotypes, thus they are more represented. As segment 2 and 6 both encode for outer shell proteins, perhaps there is a fitness advantage if they remain together. Infection of *C. sonorensis* with one of these reassortant progeny viruses to assess whether it elicits increased replication or a shorter extrinsic incubation period would be a fascinating follow-up study.

In addition to reassortant progeny, another notable outcome was the presence of mixed genotypes or detection of both parental strains within one segment. This could occur for a number of reasons. The threat of plaque bleed over, in which two plaques may be close together and appear as one plaque, could cause a mixed genotype result. Although BTV is considered to have selective segment packaging, this has not been thoroughly characterized and there could possibly be anomalous segment packing. A recent finding indicates that BTV can egress from both mammalian and insect cells in lysosome-derived extracellular vesicles.[48] If extracellular vesicles contain both BTV-10 and BTV-17 progeny virus that infects a single cell this could confound the plaque isolation approach for identifying reassortant progeny and also result in the observed mixed genotype. Egress and infection via extracellular vesicles could potentially confound accurate quantification of BTV via plaque assays or TCID50 and warrants further investigation. This may be reflected in the experimentation variation that Ramig et al. observed in BTV reassortment studies.[29] Potential for plaque bleed over and egress by extracellular vesicles demonstrate the limitations of using plaque isolation to distinguish progeny virus. Another challenge is plaque bias. Plaque bias can occur if the cell type used to isolate plaques is preferentially infected by and demonstrates cytopathic effect of certain genotypes of progeny virus.

Thus, the results of this study highlight the complexities of BTV reassortment and the inherent challenges in its investigation. The complexities include considering the several requirements of reassortment such as segment packaging, replicative compatibility, and fitness, when interpreting results. Hopefully as new molecular technologies progress, such as single cell sequencing and reverse genetics, research platforms will emerge that will facilitate the investigation of the complex evolution of impactful infectious diseases such as BTV.

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CHAPTER 4 – IN SITU HYBRIDIZATION (RNAscope®) DETECTION OF BLUETONGUE VIRUS SEROTYPE 10 AND 17 IN EXPERIMENTALLY COINFECTED *CULICOIDES SONORENSIS*

Introduction

Bluetongue virus (BTV) is an arbovirus listed by the World Organisation for Animal Health (The OIE) that can infect both domestic and wild ruminants, with sheep being particularly susceptible. As an animal disease of international concern, there can be severe economic consequences to livestock stakeholders through production loss and trade restrictions.[1,2] The arthropod culpable for disseminating BTV is the *Culicoides* biting midge (Diptera: Ceratopogonidae) with the geographical range of BTV defined by the presence of competent *Culicoides* species.[3,4] Concerningly, there have been recent incursions of novel serotypes of BTV into historically enzootic regions include the Southeastern United States (US), South America, Israel, and Australia.[5–9] Explanations for these observed expansions include increased vector distribution secondary to climate variability and virus evolution resulting in altered transmission dynamics.[10–12]

Evolution of BTV is of particular interest as it is a segmented double stranded RNA virus that is taxonomically in the order *Reovirales*, family *Sedaroviridae,* and genus *Orbivirus.*[13–15] The serotype is defined by the viral protein two outer capsid structure (VP2) encoded by nucleic acid segment two segment two that elicits the antibody response in the livestock host.[16,17] There are currently > 29 recognized bluetongue serotypes.[18–20] As BTV has a segmented genome, if a cell is infected with two different parental virus strains, evolution via reassortment is possible.[21,22] Reassortment of BTV has been characterized abundantly in field surveillance

sequencing.[23,24] However, BTV dissemination, tissue tropism, and the extent of cellular coinfection occurrence in the midge host are largely unknown. Application of in situ hybridization (ISH) technology could advance knowledge of virus presence within the midge host tissues. ISH technology amplifies the signal of specific nucleic acid sequences, permitting the visualization of nucleic acid at the cellular level of, including those of pathogens, within a histological section.[25,26]

In this study we applied RNAscope® technology (a commercial in situ hybridization platform by Advanced Cell Diagnostics [ACD]) to *Culicoides sonorensis* exposed by bloodmeal to two different BTV serotypes, BTV serotype 10 (BTV-10) and BTV serotype 17 (BTV-17) to characterize virus tissue tropism and potential for cellular coinfection. RNAscope® probes designed for segment two of BTV-10 and BTV-17 were applied to histological cross-sections of BTV exposed *Culicoides sonornesis*. Overall, there was success in detecting the presence of BTV-10 and BTV-17 via this technique and interesting patterns in tissue tropisms and localizations were observed.

Materials and Methods

Culicoides sonorensis and viruses

Infection studies of *C. sonorensis* were accomplished using the AK colony provided by the USDA ARS, Manhattan, KS, USA. This colony is derived from wild midges collected in Idaho in 1973.[27] Upon arrival, *C. sonorensis* were acclimatized for at least 24 hours at 25°C ± 2°C on a 12:12 hr light cycle and fed ad libitum 10% sugar water.[27,28]

BTV-10 (Bluetongue virus, type 10, strain 8, ATCC®VR-187™) and BTV-17 (BTV serotype 17 CO 2018) viral strains were used for the infections of *Culicoides sonorensis*. BTV-10 was acquired from an ATCC strain isolated from a sheep in California in 1952 and passaged

eight times on BHK21 cells.[29] The BTV-17 strain was isolated from the whole blood of a sheep in Colorado in 2018 on CuVaW3 cells and subsequently passaged 2 times on BHK21 cells.[30]

C. sonorensis infection and maintenance

Sheep blood defibrinated mechanically (Hemostat Laboratories, Dixon, CA, USA or Lampire Biological Laboratories, Everett, PA, USA) was screened the for presence of BTV and antibodies via qRT-PCR and cELISA (VMRD, Pullman, WA, USA). Single BTV-10 or BTV-17 infections and BTV-10 and BTV-17 coinfections were established via virus-spiked blood meals.[31] For each single infection and coinfection treatment group, BTV stock was diluted in defibrinated sheep blood to produce a final titer of 1 x 10^5 $TCID_{50}$/mL (Table 4.1). For the negative control group, defibrinated sheep blood was combined with inoculum media of the same volume as that used in the infectious blood feeding treatment groups. *C. sonorensis* were infected with BTV-spiked blood 3-4 days post-emergence. Glass bell feeders equipped with parafilm membranes, were used to supply 37°C prepared blood treatments. Midges were given access to prepared blood for 80 minutes. After blood feeding, *C. sonorensis* were immobilized by placement into a -20°C freezer for 5 minutes. Blood fed *C. sonorensis* were then sorted on a modified chill table and retained for the study by grouping approximately 100 *C. sonorensis* per container.

For the remainder of the study, *C. sonorensis* were housed in non-treated paper tube containers (Rigid Paper Tube Corporation, Wayne, NJ, USA) with sheer panty hose stretched over the lid to provide air exchange and a mechanism for feeding. Sugar water (10% *w/v*) was available at all times through a cotton wick placed on each container. *C. sonorensis* were maintained at 25°C ± 2°C with a 12:12 hour light cycle.

Table 4.1 - BTV $TCID_{50}$/mL In Blood Meal for Infection Groups.

Tx Group	BTV-10 $TCID_{50}$/mL	BTV-17 $TCID_{50}$/mL	Total $TCID_{50}$/mL
Negative blood meal	N/A	N/A	N/A
BTV-10 blood meal	1×10^5	N/A	1×10^5
BTV-17 blood meal	N/A	1×10^5	1×10^5
BTV-10 & BTV-17 blood meal	5×10^4	5×10^4	1×10^5

Day ten post-infection, *C. sonorensis* were collected for processing. Treatment groups were placed in CellSafe capsules inserted in biopsy cassettes and then immersed in 10% buffered formalin. *C. sonorensis* were embedded in paraffin, sectioned, and mounted on SuperFrost Plus Slides by the Colorado State University Biopsy and Histopathology Service Department as described by the ACD Formalin-Fixed Paraffin-Embedded (FFPE) Sample Preparation and Pretreatment for the RNAscope® 2.5 Assay (Document Number 322452).

In Situ Hybridization: RNAscope® 2.5 HD Chromogenic Duplex Detection

Chromogenic visualization of BTV-10 and BTV-17 nucleic acid was accomplished with RNA in situ hybridization (RNA-ISH) technology using the commercially marketed RNAscope® *2.5 HD Chromogenic Duplex Detection Kit (Chromogenic) (Advanced Cell Diagnostic ACD).* Sequences for all 10 segments of BTV-17 and BTV-10 used in this study were provided to ACD for probe design targets and to make sure probes do not cross react with other BTV segments (Table 4.2). The nucleic acid segment two that encodes for VP2 of BTV-17 (nucleotides 1801-2839, OQ798199, with ability to cross react with MT95297 of BTV-17 California 1988) is the target for the Channel 1 (C1) probes and of BTV-10 (nucleotides 184 - 1168; GenBank accession number JQ740772 with the ability to cross react with MW456748 BTV-10 California 1952) is the target of the Channel 2 (C2) probes. The positive control probes designed for Channel 1 targets the protein elongation actor 1-beta (*EF1b* nucleotides 2-954;

GenBank accession number GAWM01010754) that has demonstrated high expression across different conditions and demonstrated high CT values in diverse tissue types.[32] The positive control probes designed for Channel 2 targets the vacuoloar ATPase gene (nucleotides 2-668; GenBank accession number AY753855.1) that has been sequenced from the same AK *C. sonorensis* colony and has been applied as an internal positive extraction control for previous *Culicoides sonorensis* studies.[33,34] The dihydrodipicolinate reductase (*DapB*) gene, provided by ACD, was utilized as a negative control and to evaluate for non-specific staining as it was expected to be absent in study samples. A table of probe targets is provided (Table 4.3). All probes were designed and manufactured by Advanced Cell Diagnostics. Probes were designed to not cross react with each other, other segments of BTV strains used in the study, or known *C. sonorensis* sequences.

Table 4.2 - GenBank Accession # For BTV-17 & BTV-10 Segments.

Nucleic Acid Segment	BTV-17 CO 2018	BTV-10 ATCC CA 1952
1	OQ798198	MW456747
2	OQ798199	MW456748
3	OQ798200	MW456749
4	OQ798201	MW456750
5	OQ798202	MW456751
6	OQ798203	MW456752
7	OQ798204	MW456753
8	OQ798205	MW456754
9	OQ798206	MW456755
10	OQ798207	MW456756

Table 4.3 – Probe Targets.

Probe	Probe Description	NCBI sequence	Sequence bp range
Negative	DapB	ACD provided	ACD provided
Positive Control C1	Cso-TSA-m41748	GAWM01010754	2-954
Positive Control C2	Cso-V-ATPase16	AY752855	2-668
C1	V-BTV17-segment2-VP2-C1	OQ798199	1801-2839
C2	V-BTV10-segment2-VP2-C2	JQ740772	184 - 1168

One slide from each of the *C. sonorensis* treatment groups was stained with haematoxylin and eosin to evaluate cross-sections and assist in identifying anatomical structures. Sample preparation, pretreatment and staining procedures were conducted according to the Formalin-Fixed Paraffin-Embedded (FFPE) Sample Preparation and Pretreatment for the RNAscope® 2.5 Assay Part 1 (Document Number 322452) and RNAscope® 2.5 HD Duplex Detection Kit (Chromogenic) Part 2 (Document Number 322500-USM) with probes, reagents, and the HybEZ™ II Oven recommended by ACD.

Slide Pretreatment. Slides were dried for 1 hr at 60°C, deparaffinized in xylene twice for 5 min, washed twice in 100% alcohol for 1 min, and then dried for approximately 5 minutes. Tissue sections were covered with hydrogen peroxide and incubated for 10 minutes. Hydrogen peroxide was removed from the slide and dipped in distilled water several times to remove any remaining reagent. The slides were boiled at 100°C for 15 minutes in beakers containing the target retrieval reagents After rinsing with distilled water, slides were immersed in100% ethanol for 3 minutes, followed by a drying period of approximately 5 minutes. RNAscope® Protease Plus was applied for 15 min at 40°C.

Staining. Excess liquid was removed from slides and 4 drops of the probe mix (C1: BTV-17 and C2: BTV-10) was applied to treatment group slides including negative control group. 4 drops of control probes (Negative: DapB; positive control C1: Cs0 -TSA-m4178; positive control

C2: Cso-V-ATPase16) were applied to separate control slides to affirm the success of the hybridization procedure. After application of probes, slides were incubated for 2 hr at 40°C.

Hybridization. Signal amplification scaffolds were synthesized through a series of hybridization steps. Hybridization steps 1-6 were completed for detection of the red signal. To create the green signal, hybridization steps 1-10 were performed (Table 4.4). Steps 1 through 6 entailed washing each slide in 1X Wash Buffer followed by the addition of 4 drops of the corresponding AMP reagent to each slide. Slides were incubated at 40° (30 min for AMP1, 15min for AMP2, 30 min for AMP 3, 15 min for AMP4) or incubated at room temperature (30min for AMP5 and 15 min for AMP6). Detection of the red signal, which detects the C2 probe (BTV-10) was accomplished by application of a 1:60 ratio of Red-B to Red-A reagents to each slide for an incubation period of 10 min at room temperature. Afterwards, the slides were washed twice in 1X Wash Buffer in preparation for hybridization steps 7 - 10 for green signal detection.

For hybridization steps 7-10, 4 drops of the corresponding AMP reagent were applied to each slide, and then incubated at 40° C (15min for AMP7, 30min for AMP8) or incubated at room temperature (30 min for AMP9 and 15min AMP10). After each hybridization step, the slides were washed twice in 1X Wash Buffer. Detection of the green signal, which detects the C1 probe (BTV-17), was accomplished by application of a 1:50 ratio of Green-B to Green-A reagents to each slide for an incubation period of 10 min at room temperature followed by two washes in 1X Wash Buffer. Slides were counterstained in 50% Gill's hematoxylin for 30 sec., To remove excess stain, slides were immersed in tap water several times and incubated for 15 minutes at 60°C to dry. After 5 min of cooling at room temperature, slides were dipped into

xylene briefly, then mounted using VectaMount Mounting Medium after which a coverslip was placed over the section.

Table 4.4 – Hybridization and Signal Detection Steps

Hybridization reagent	Incubation Temp	Incubation Time
AMP 1	40° C	30min
AMP 2	40° C	15min
AMP 3	40° C	30min
AMP 4	40° C	15min
AMP 5	Room Temp	30min
AMP 6	Room Temp	15min
C2 (Red) Signal detection	Room Temp	10min
AMP 7	40° C	15min
AMP 8	40° C	30min
AMP 9	Room Temp	30min
AMP 10	Room Temp	15min
C1 (green) Signal detection	Room Temp	10min

Image capture

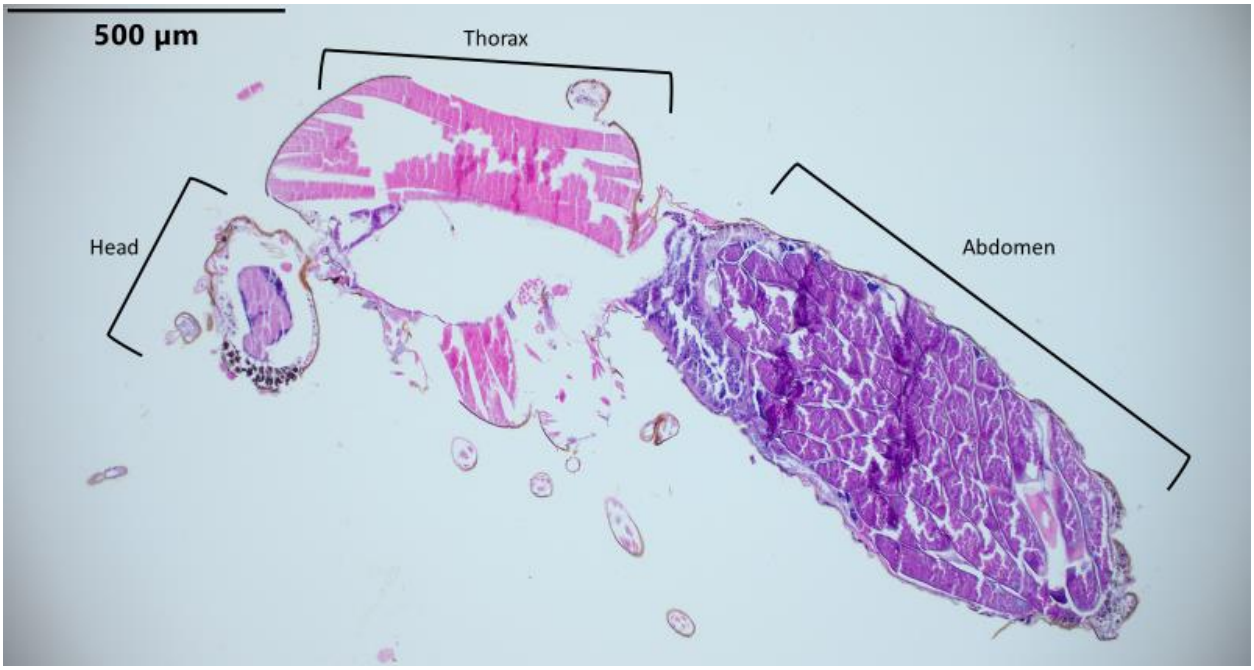
Images of histological slides were visualized on an Olympus BX53 microscope and captured with an attached Olympus DP28 Camera. Images were processed using the Olympus cellSens Entry software.

Results

Identification of C. sonorensis structures in haematoxylin and eosin sections

Haematoxylin and eosin stained slides of *C. sonoren*sis facilitated the identification of anatomical structures as a reference to evaluate BTV virus distribution (Figure 4.1). Structures routinely observed include the cerebral ganglion, ommatidia, salivary glands, dorsal longitudinal muscles, and epicuticle. Johnston organs, fatbody, tracheal cuticle, legs, midgut, ovarian follicles, and overlaying follicular epithelium are evident in some cross-sections.

A.



B.

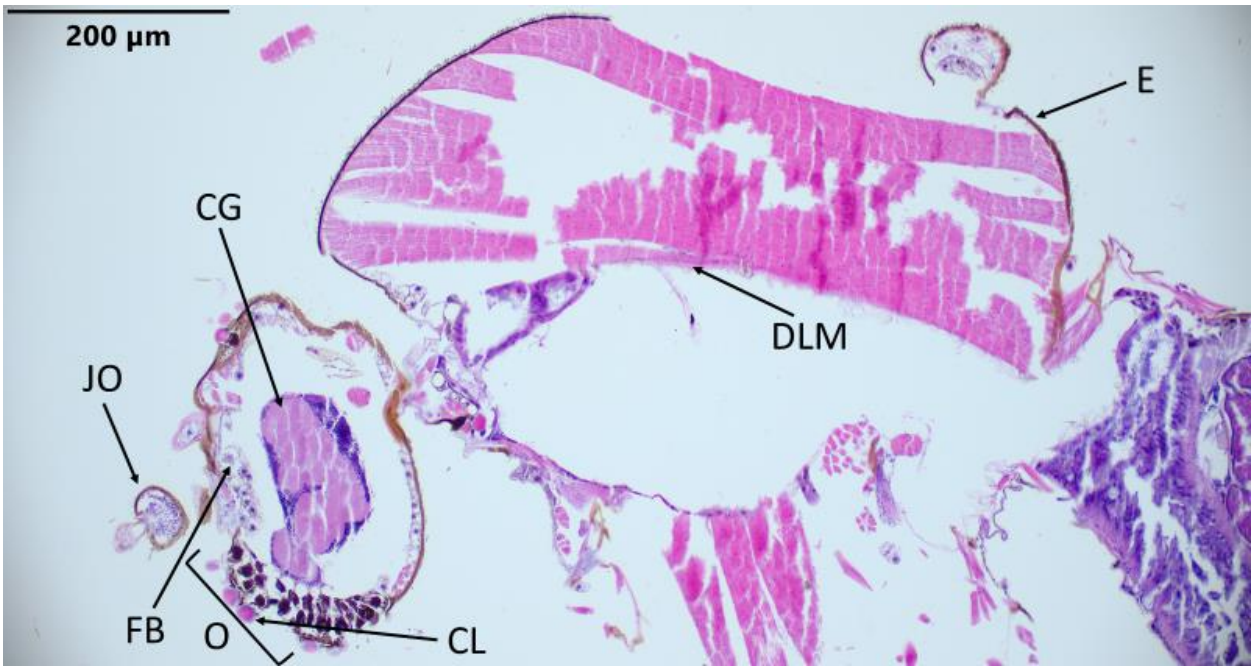


Figure 4.1 - *C. sonorensis* Sections Stained with Haemotoxylin and Esosin (A) *C. sonorensis* at 10x **(B)** Head and thorax at 20X. Tissues labelled: JO, Johnston's organs; O, ommatidia; CL, corneal lens; FB, fatbody; E, Epicuticle; CG, Cerebral ganglion; DLM, dorsal longitudinal muscle.

Control probes indicate successful RNAscope® hybridization and probe detection

Successful RNAscope® hybridization and probe detection was demonstrated by the application of control probes. The negative control probe designed for the DapB gene exhibited no staining with the exception of some non- specific staining of the ommatidia and the channel one and channel two positive control host probes (for detection of *EF1b* and vacuoloar ATPase housekeeping genes respectively) exhibited green and red staining respectively (Figure 4.2). None of the mock infected *C. sonorensis* exhibited staining for BTV-17 or BTV-10 with the exception of non-specific red staining of the corneal lens of the ommatidia.

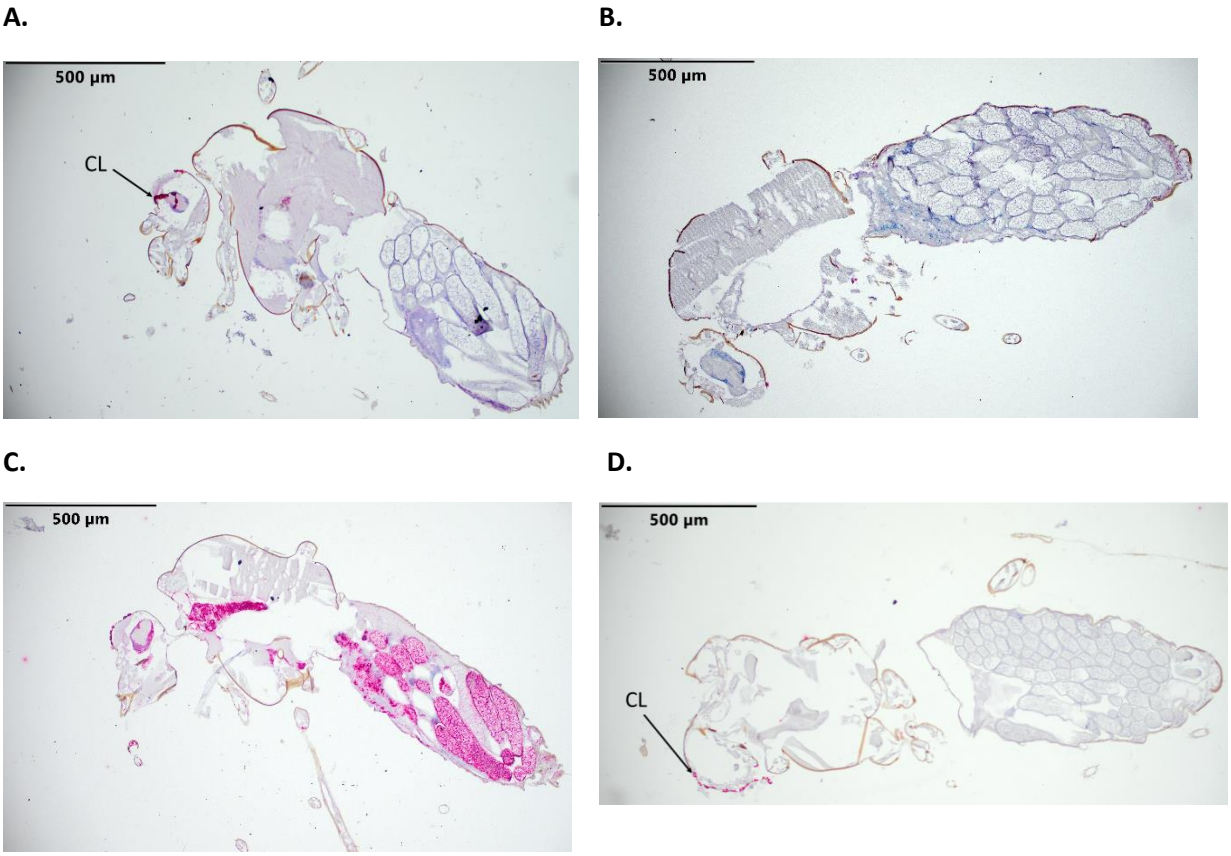


Figure 4.2 - *C. sonorensis* Sections Stained with RNAscope® Control Probes (A) Negative control probe (bacterial DapB RNA). Tissue labelled: CL, corneal lens with nonspecific staining. **(B)** Positive Channel 1 control (Cso – TSA - m4178) stained with green **(C)** Positive Channel 2 control probe (Cso – V – ATPase16) stained with Red Fast. **(D)** BTV-10 (C2) and BTV-17 (C1) segment 2 specific probes (Mock-infected)

Detection and distribution of BTV-10 and BTV-17 single infection treatment groups indicate increased prevalence and dissemination in BTV-17 exposed C. sonorensis

C. sonorensis fed a blood meal of BTV-17 had a staining detection of 61.5% (Table 4.5). All specimens with detection of BTV-17 had virus dissemination beyond the midgut. Presence of BTV-17 CO was notable in mouth parts, basal areas of the ommatidia, salivary gland, epicuticle, and midgut. There is non-specific red staining of the corneal aspect of the ommatidia. (Figure 4.3).

Table 4.5 - % of *C. sonorensis* with Detectable BTV-10 and BTV-17 Segment 2

Tx Group	Detection of BTV-10 segment 2 only	Detection of BTV-17 segment 2 only	Detection of BTV-10 & BTV-17 segment 2	No detection
BTV-10	13.30%	0%	0%	86.70%
BTV-17	0%	61.50%	0%	38.50%
BTV-10 & BTV-17	7.10%	21.40%	21.40%	50%

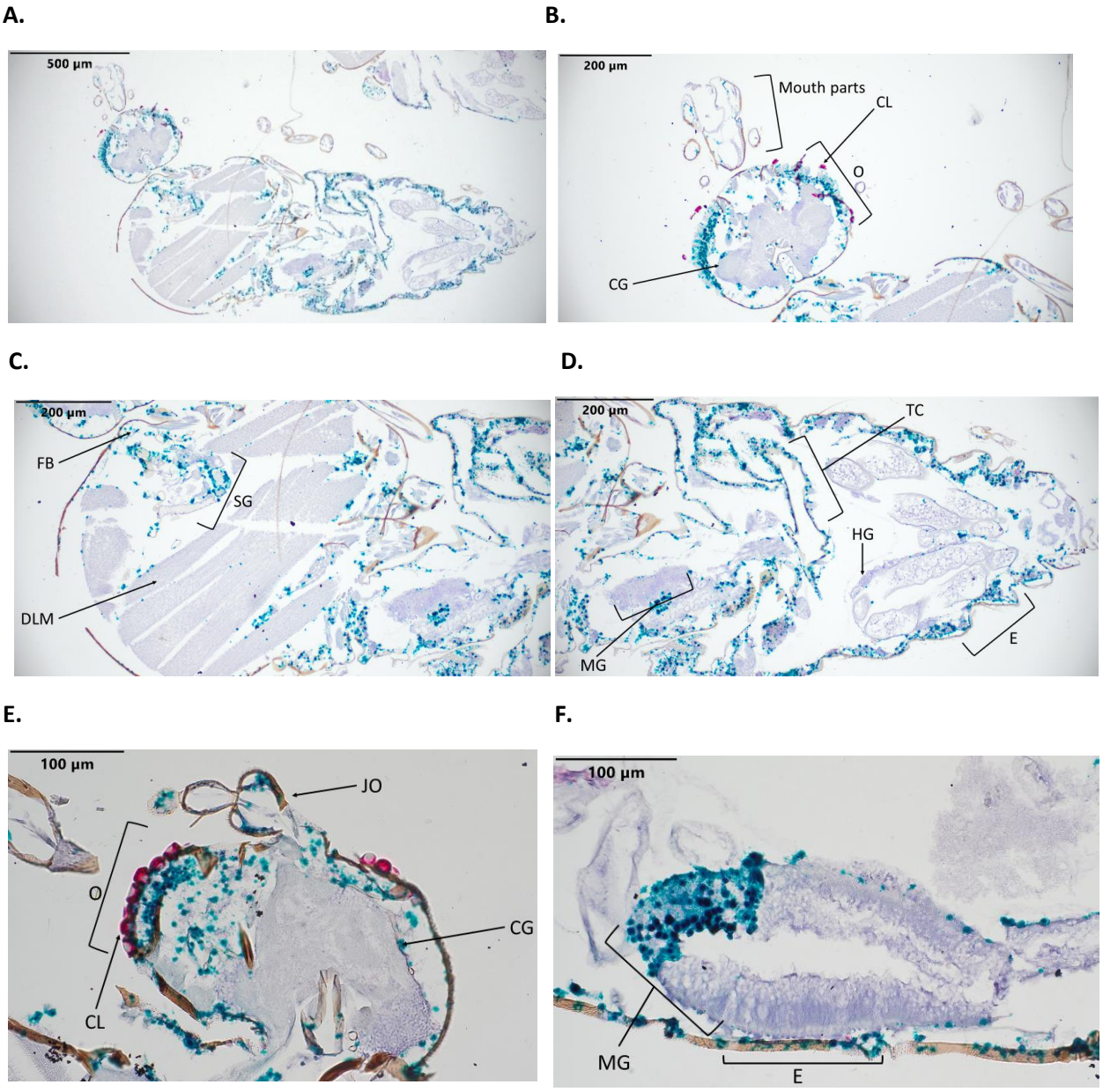


Figure 4.3 - *C. sonorensis* 10 Days Post BTV-17 Bloodmeal Sections. Stained with BTV-10 and BTV-17 segment 2 Probes. BTV-10 probe (Channel 2) binding is red staining and BTV-17 probe (Channel 1) binding is green staining. (**A**) Full body at 10x **(B)** Head at 20x **(C)** Thorax at 20x **(D)** Abdomen at 20x. **(E)** Head at 40x. **(F)** Abdomen at 40x. Tissues labelled: JO, Johnston's organ; O, ommatidia; CL, corneal lens; CG, cerebral ganglion; FB, Fatbody; SG, salivary gland; DLM, dorsal longitudinal muscle; E, Epicuticle; TC, tracheal cuticle; MG, midgut; HG, hindgut.

C. sonorensis fed a blood meal of BTV-10 had a staining detection of 13.3% (Table 4.5).

There is evidence of dissemination of BTV-10 in some of the specimens with presence of BTV-

10 evident in the epicuticle. In gravid females BTV-10 was present in the follicular epithelium, but absent from the ovarian follicles (Figure 4.4). However, there was a specimen that had most of the virus localized to the midgut, with intensive staining (Figure 4.4 C and D).

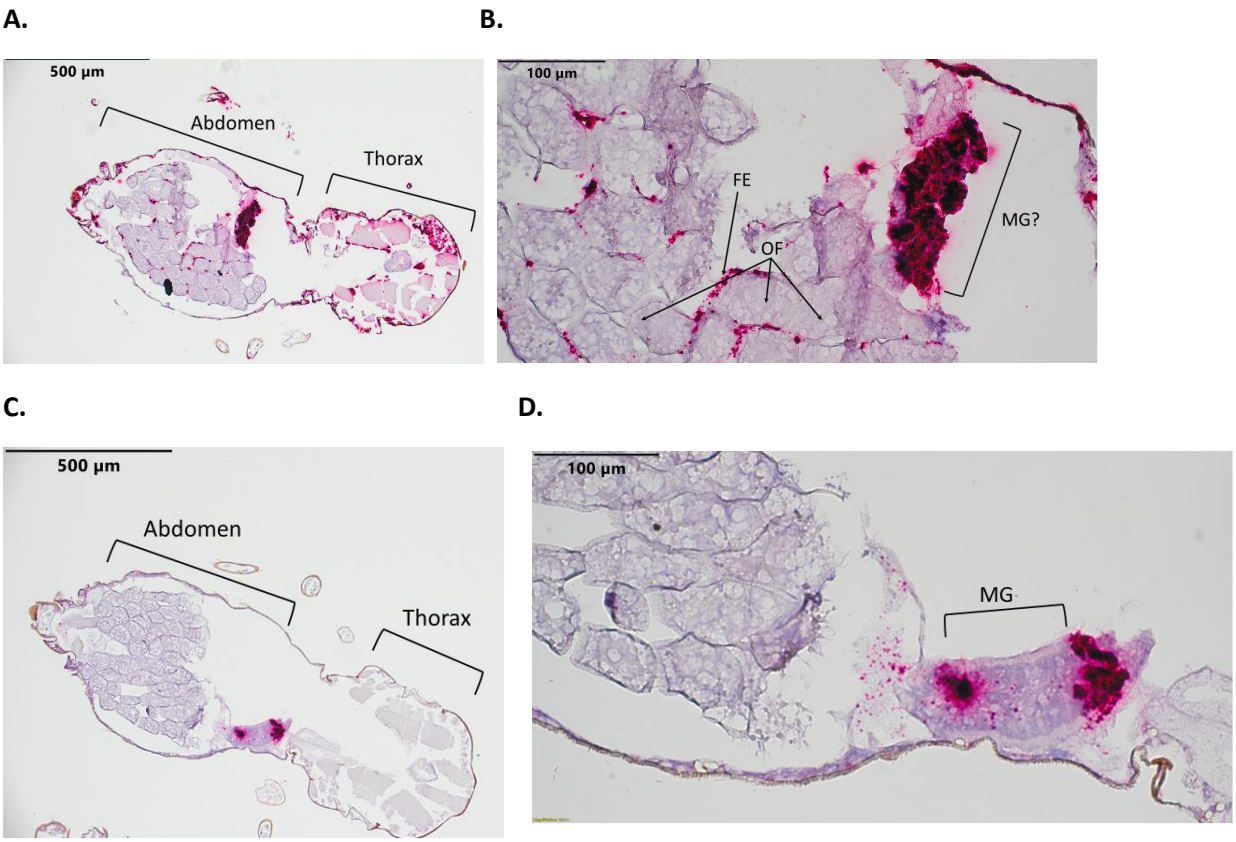


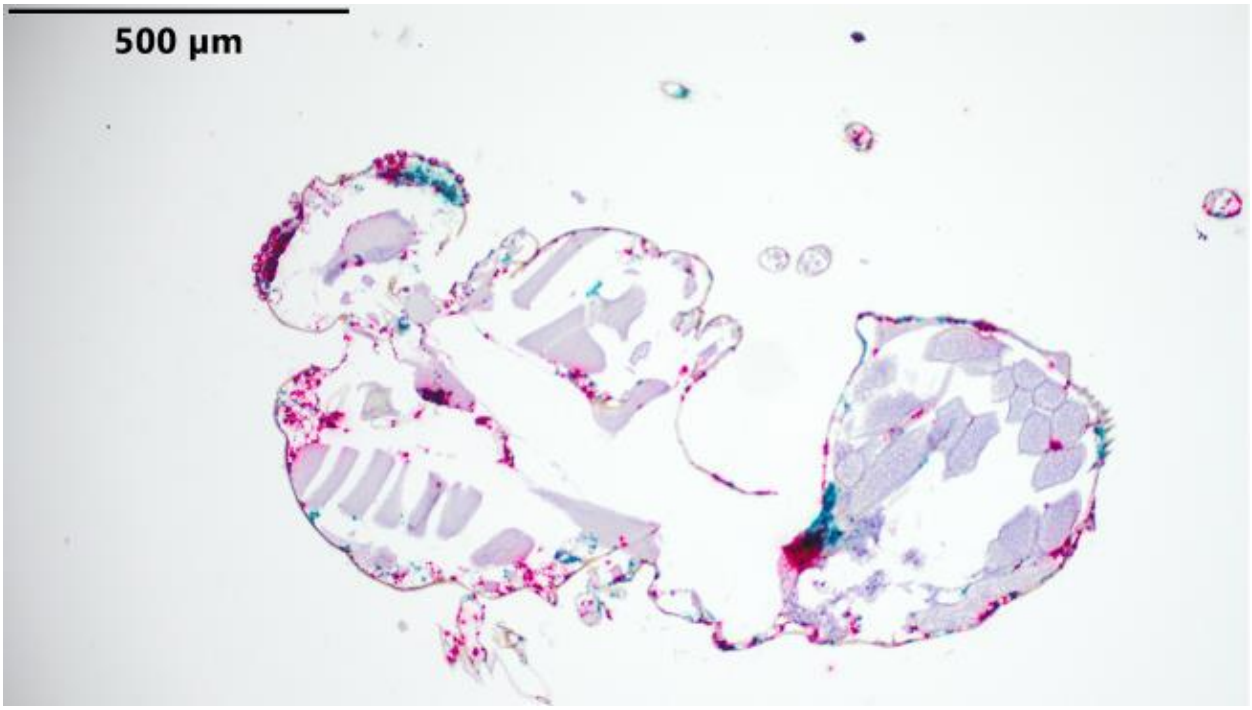
Figure 4.4 - *C. sonorensis* 10 Days Post BTV-10 Bloodmeal Sections. Stained with BTV-10 and BTV-17 segment 2 probes. BTV-10 probe (Channel 2) binding is red staining and BTV-17 probe (Channel 1) binding is green staining. (**A**) Full body at 10x (**B**) Abdomen at 40x **(C)** Full body at 10x with limited BTV dissemination. **(D)** Same specimen from (C) abdomen at 40x. Tissues labelled: MG, Midgut; OF, ovarian follicles; FE, follicular epithelium.

Detection and distribution of BTV-10 and BTV-17 coinfection indicate similar tissue tropism, but modest colocalization of serotypes.

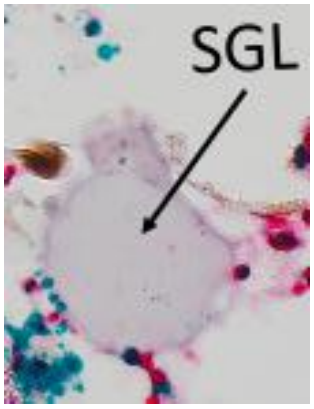
Presence of both BTV-17 and BTV-10 were present in 21.4% of midges while 21.40% only exhibited BTV-17 and 7.10% only exhibited BTV-10 (Table 4.5). Individual *C. sonorenesis* demonstrated varied patterns of BTV-10 and BTV-17 distribution. *C. sonorensis* Figure 5A shows evidence of BTV-10 and BTV-17 dissemination. In contrast, *C. sonorensis* figure 5B

demonstrates dissemination of BTV-17 with BTV-10 mostly confined to the midgut (unfortunately the head was not captured in the cross-section, demonstrating the challenges in performing histology on such small specimens). Figure 4.5C is an example of detection of BTV-17 only. Interestingly, while BTV-17 and BTV-10 demonstrate similar tissue tropism, they often present in a mosaic pattern with limited overlapping (Figure 4.5). Tissues with virus detection included ommatidia, cerebral ganglion, salivary gland (including a small presence of both BTV serotypes in the salivary lumen) fatbody, midgut, and epicuticle. For both serotypes, BTV was apparent in the follicular epithelium, but absent in the ovarian follicles.

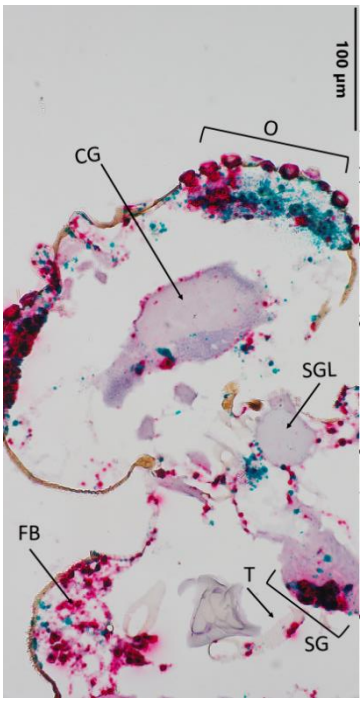
A.



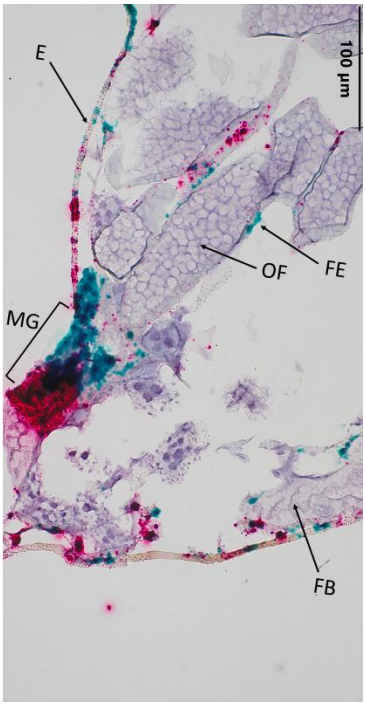
Salivary gland lumen



Head



Abdomen



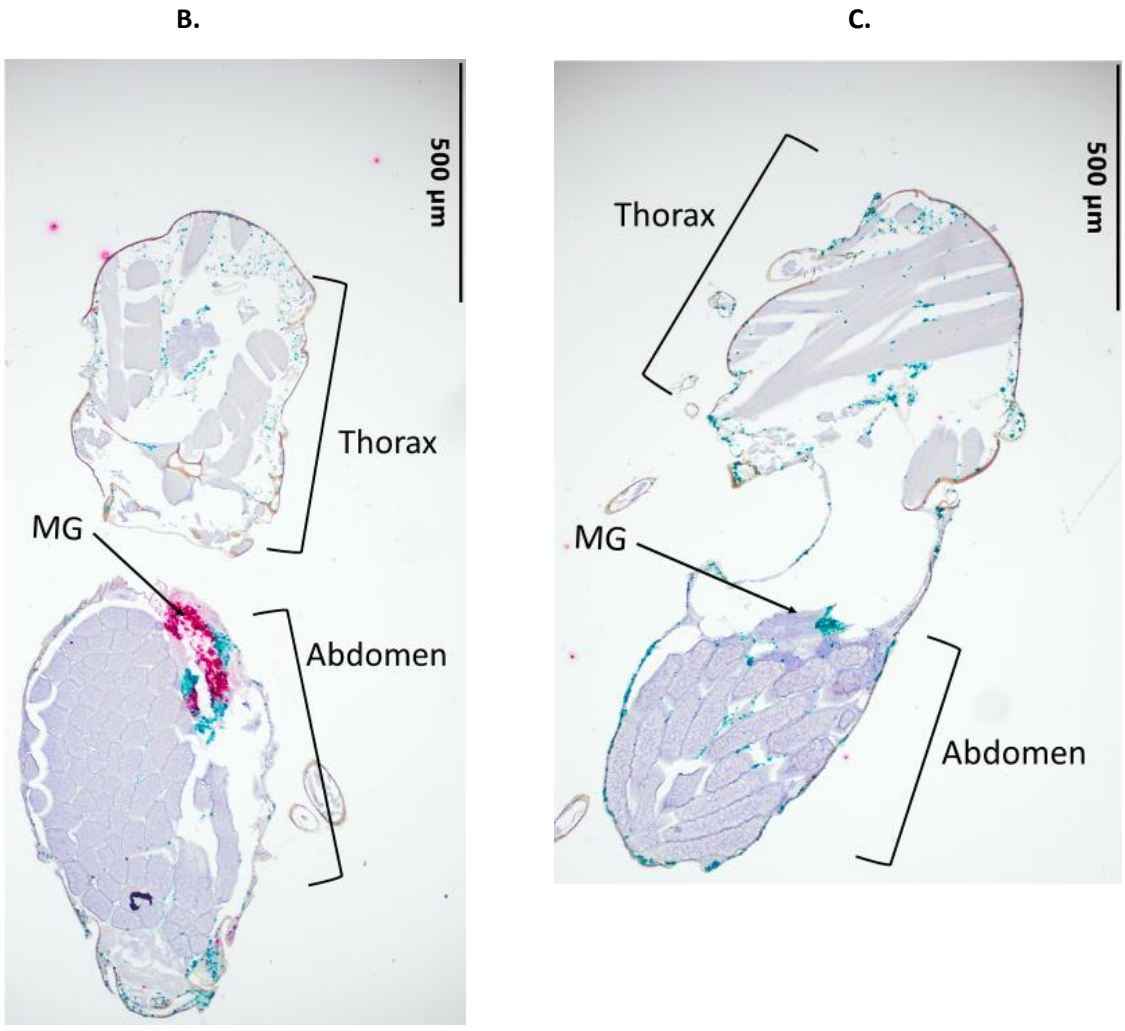


Figure 4.5 - *C. sonorensis* 10 days Post Combined BTV-10 and BTV-17 Bloodmeal Sections. Stained with BTV-10 and BTV-17 segment 2 specific probes. BTV-10 probe (Channel 2) binding is red staining and BTV-17 probe (Channel 1) binding is green staining. (**A**) Disseminated BTV-10 and BTV-17CO infection at 10x with salivary gland lumen, head, and abdomen at 40x (**B**)Disseminated BTV-17CO infection with BTV-10 ATCC localizing to midgut at 10x **(C)** Disseminated BTV-17CO with BTV-10 absent at 10x. Tissues labelled: O, ommatidia; CG, cerebral ganglion; FB, fatbody; T, trachea; SGL, salivary gland lumen; SG, salivary gland; Epicuticle;; CL, corneal lens; E, epicuticle' MG, midgut; OF, ovarian follicle; FE, follicular epithelium.

Discussion

This study demonstrates the capabilities of a chromogenic duplex in situ hybridization (RNAscope®) platform to detect the distribution of two different BTV serotypes in a coinfected

C. sonorenesis in vivo model. In *C. sonorensis* exposed to both BTV-10 and BTV-17, it is important to interpret probe detection in context of potential reassortment as the probes only detect nucleic acid of segment two from each serotype. Thus, while segment two is detected, the corresponding virion could potentially have nucleic segment contributions for the remaining nine segments from either parental strain. As BTV has the potential to evolve drastically via reassortment, understanding viral dissemination and cellular coinfection in the BTV *C. sonorensis* vector may inform predictive modeling and enhance understanding of reassortment mechanisms and barriers.

Important BTV dissemination barriers of *C. sonorensis* have been identified as the mesenteron infection barrier (prevents BTV infection of midgut), mesenteron escape barrier (limits BTV to gut cells) and a dissemination barrier (prevents BTV in the haemocoel from infecting other tissues).[35] BTV-10 in both single and coinfected midges occasionally had examples of specimens with BTV-10 detection being limited to the midgut without dissemination (Figure 4.4C, 4.4D, and 4.5C). Detection of BTV-10 and BTV-17 segment two in the coinfected midges demonstrated a variety of dissemination outcomes with BTV-17 segment two overall dominating the dissemination landscape of *C. sonorensis*. Perhaps this segment (or corresponding strains/reassortants) confers an advantage within the midge host while BTV-10 has challenges at the mesenteron escape barrier.

The detection of BTV in the salivary glands is notable as this is the route which BTV is transmitted to the ruminant host (Figure 4.3C and 4.5A). Currently, a salivary gland infection barrier and a salivary gland escape barrier for BTV has not been recognized in the midge.[35] In these cross-sections, a small amount of virus was detected in the lumen of the salivary gland. It would be curious if the act of feeding stimulates the release of virus into the salivary gland

lumen which is then transmitted to the mammalian host via the blood meal. Previous studies on the ultrastructure of *Musca domestica* salivary glands infected with hypertrophy virus indicated that there is a cuticular, chitinous lining between the secretory cells and salivary duct lumen that may require disruption for virus to access the lumen and actually act as an exit barrier for the virus. Hypertrophy virus does result in pathological degradation of this cuticle barrier permitting the release of the virus. It would be expedient to investigate if this is a similar mechanism for BTV infected midges and if there is time dependency to this mechanism that would affect transmission to the ruminant host.[36]

When both BTV-10 and BTV-17 segment two were present, there was mostly a mosaic staining pattern suggesting that cellular coinfection between serotype 10 and 17 at 10 days post infection is not extensive. This could be due to the occurrence of super infection exclusion in which once an infection has been established in a cell it limits entry of a second virus. It has been demonstrated that BTV can spread cell to cell as free virus particles or via extracellular vesicles containing numerous virus particles.[37] While extracellular vesicles are more efficient at establishing infection, the free virus particles are able to overcome super-infection exclusion easier. If the majority of BTV in the infectious bloodmeal were in extracellular vesicle form, this would result in reduced occurrence of initial cellular coinfection in the *C. sonorenesis* vector. Furthermore, if BTV egress from *C. sonorenesis* cells is also as extracellular vesicles, this would result in reduced occurrence of cellular coinfection and explicate the mosaic patterns between serotypes observed in coinfected *C. sonorenesis.* However, an ISH fluorescence platform could be employed for better resolution in evaluating cellular coinfection.

Detection of virus in single infection samples indicated that both virus strains had similar tissue tropisms and were routinely detected in the ommatidia, brain, fat bodies, trachea, and the

epicuticle. While pathological changes and metabolic alterations are difficult to assess in invertebrates via histology, the presence of virus in these tissues may provide a connection to understand changes in the infected insect. Indeed, notable changes in insects infected with virus have included alterations in midgut lipid metabolism in *Aedes aegypti* infected with dengue virus, deviations in foraging behavior *of Solenopsis invicta* infected with Solenopsis invicta virus 3, and altered susceptibility of *Drosophila melanogaster* to certain bacterial and fungal pathogens that are infected with galbut virus.[38–40] Ichnovirus from *Hyposoter didymator* induces apoptosis in fat body cells of *Spodoptera frugiperda* larvae, resulting in modulated immunity.[41] As more life trait and metabolome studies elucidate the changes occurring in BTV infected *C. sonorensis*, knowledge of BTV tissue tropism will assist in understanding the pathogenesis.

Similar to previous insect in situ hybridization studies, *C. sonorensis* specimens demonstrated non-specific red staining of the ommatidia.[25] Thus, caution must be paid when interpreting viral tropism for eyes when using this stain. However, abundant staining in infected midges compared to the negative controls and presence of the green signal for BTV-17 in exposed midges indicate that there is viral tropism for the ommatidia. Prior IHC BTV studies also confirm a BTV tropism for *C. sonorensis* ommatidia.[42] Viral tropism for insect ommatidia is evident in other arthropod borne viruses including Rift Valley virus.[25] As BTV has been detected in the ommatidia of the midge, there could possibly be an effect on midge visual cues which have implications for trapping and insect surveillance techniques. In addition to detecting BTV in the ommatidia, it has been suggested that midges with BTV may have light aversion as compared to midges without BTV. A higher proportion of midges with BTV detected where found in CO_2 only traps compared to CO_2 with lights (CDC insect traps).[42] Additionally, detection of virus in the ganglia could play a part in altering midge behavior.

Absence of viral detection in the midge ovarian follicles among all infection groups is consistent with current understanding that BTV is not transmitted vertically in the invertebrate host.[43] While there seems to be abundant virus in the epithelium overlaying ovarian follicles, there is not detection of the virus within. While we were curious to investigate the presence or absence of virus detection in gravid midges to further validate vertical transmission studies, more adequate visualization of other abdominal structures (including more complete midgut and Malpighian tubules) may be accomplished with *C. sonorensis* not containing abundant ovarian follicles.

Challenges of the study included consistently obtaining full cross sections of the small *C. sonorensis* and identifying appropriate *C. sonorensis* housekeeping gene sequences for control probes. *C. sonorensis* is substantially smaller at an average body length of 1.5 mm than its *Culex pipiens* mosquito counterpart with an approximate length of 3mm.[25,34] However, an advantage to the in situ hybridization technique is that it facilitates detection of virus in different tissues as dissection and isolation of distinct tissue types can be very limited and challenging in such a small insect. Initial attempts at designing a positive control probe using the COX1 housekeeping gene that we use for PCR was deemed not feasible by RNAscope Engineers due to its short nucleotide. Our positive probe design targets were informed by work that indicated the selected targets were reliably expressed.[32] GAPDH sequence is another potential target for positive control design that is often used in mosquito work, however a sequence specific to *Culicoides spp* could not be identified in GenBank at this time.[25]

In situ hybridization (RNAscope®) platform has been successfully employed in *C. sonorensis* that have been coinfected with two different BTV serotypes via bloodmeal. This approach has potential to facilitate the interrogation of BTV tissue tropism, dissemination, and

cellular coinfection dynamics in the BTV insect vector to further understand the ramifications of reassortment and evolution of this virus of high consequence.

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CHAPTER 5 – CONCLUSION

BTV is a pathogen of veterinary significance with recent incursions of novel serotypes causing devastation to livestock and economic loss for stakeholders. Transmitted by the *Culicoides* biting midges, the impetus for these incursions is probably multifactorial including increased vector distribution secondary to climate variability and virus evolution. Evolution of BTV is of particular interest as it is a segment double-stranded RNA virus capable of reassortment. Reassortment can elicit large changes in genotypic diversity that may be reflected in phenotypic alterations in transmission dynamic, the ability to infect the hots, and pathogenicity. The objective of this dissertation work was to characterize the effects of coinfection with BTV-10 and BTV-17 in *Culicoides sonorensis*.

Prior studies demonstrated increased BTV virogenesis in the *C. sonorensis* in relation to warmer temperatures. However, temperature effects on reassortment has not been investigated in this disease system. It was hypothesized that BTV coinfected *C. sonorensis* maintained at higher temperatures would generate higher frequency of reassortment than at cooler temperatures. However, most progeny plaques ended up aligning with BTV-17 across all temperature groups. A small number of progeny plaques aligned with BTV-10 or had segments that aligned with both parental serotypes. This result indicated that a more fit parental serotype may out compete its coinfecting counterpart and reduce the opportunity for reassortment to occur. However, temperature did have effects on *Culicoides* survival and virogenesis rates which would impact transmission dynamics.

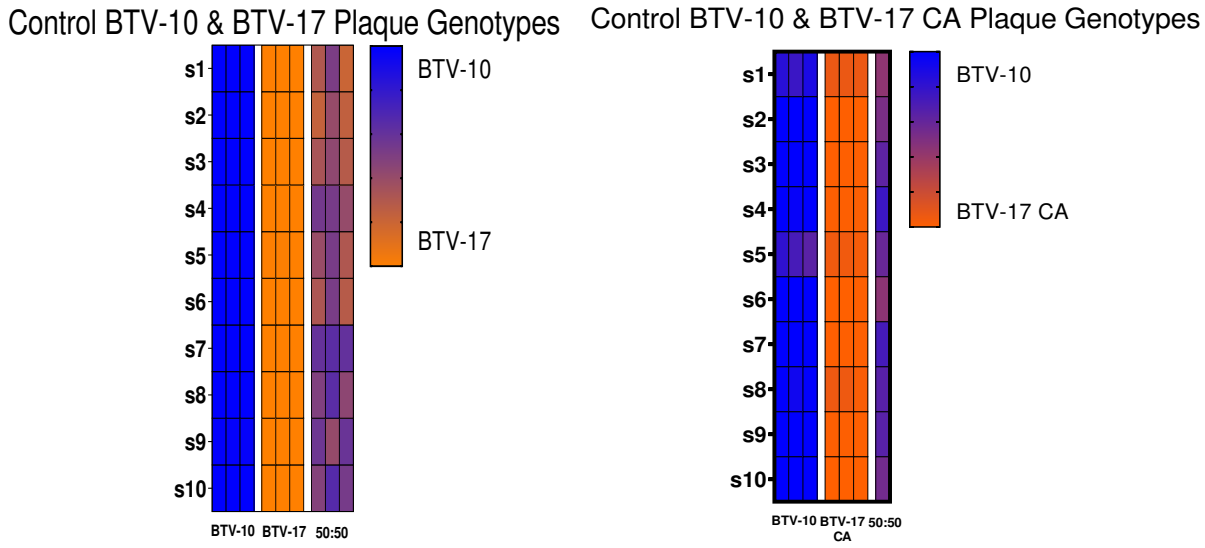
As one parental serotype dominated the BTV coinfection landscape, it was posited that using different coinfection titers may assist in leveling out the competition between parental

serotypes. Indeed, earlier *in vitro* studies indicated that the BTV parental strain with the higher multiplicity of infection contributed more genomic segments to progeny virus. Overall, there was a trend of more representation from the parental serotypes with the greater titer. There were also reassortant progeny detected in some of the pools. In one pool, reassortant plaques were highly represented and had increased representation of BTV-17 in segment 2, 4, and 6 and increased representation of BTV-10 in segment 9 and 10. Perhaps this genotype confers an advantage in the *Culicoides* host and is able to outcompete the parental strains. A supplemental investigation of how genetic relatedness between coinfecting BTV serotypes affects reassortment was performed. Successful reassortment requires segment packaging and replicative compatibility, thus parental strains that are more similar genetically may be more conducive to reassortment. However, as the pairwise identity was high, it impeded genotyping of progeny plaques.

Cellular coinfection is also a requirement for reassortment. RNAscope® technology was employed to visualize colocalization of parental serotypes within the midge host. Diverse patterns of virus dissemination were visualized in different coinfection *Culicoides.* This technology has potential to help further understanding of the dynamics between virus and vector.

As vector-borne pathogens are responsible for a high proportion of disease incursions that threaten human and animal health, understanding the multiple factors that drive the emergence of arboviruses is critical for the development of improved control and prevention strategies. The insights and data generated from this research will inform ecological, molecular, and modeling principals of emerging vector-borne diseases that can be translated to similar human pathogens such as Rift Valley fever virus and Oropouche virus, particularly in the context of climate change. Principles and approaches derived from this work can be translated to emerging viral

pathogens of human concern. As consequences of climate change are more evident, climate-based models of disease risk are growing in importance.



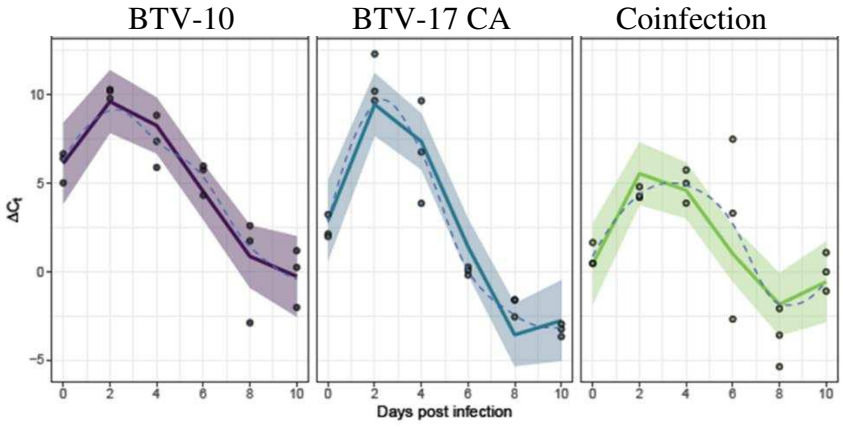
Appendix 1 – Control Plaques Genotypes. Control plaques were library prepped and sequenced with genotypes depicted by heatmaps. Control plaques for BTV-10 & BTV 17 study indicates valid sequencing runs. Control plaques for BTV-10 & BTV 17 CA study is demonstrating some BTV-17 CA reads going to BTV-10. This maybe secondary to BTV-10 and BTV-17 CA having large pairwise identities. Each column on the x -axis represents an individual plaques.The y -axis represents the ten different genomic segments of BTV. Blue indicates that 100% of the sequencing reads for that segment aligns with BTV-10. Orange indicates that 100% of the sequencing reads for that segments align with BTV-17. Color gradient in between blue and orange indicate that both parental strains aligned with the segment.

APPENDIX 2 – BTV-10 AND BTV-17 CA NUCLEOTIDE PAIRWISE IDENTITIES

Appendix 2 – Nucleotide Pairwise Identity of BTV-10 and BTV-17 CA Genomic Segments

Segment	Protein encoded	% Pairwise Identity
Seg-1	VP1	98.4
Seg-2	VP2	69.6
Seg-3	VP3	95.7
Seg-4	VP4	98.6
Seg-5	NS1	98.9
Seg-6	VP5	79.9
Seg-7	VP7	97.1
Seg-8	NS2	98.4
Seg-9	VP6/NS4	97.6
Seg-10	NS3/NS3A/NS5	82.1

APPENDIX 3 – BTV-10 AND BTV-17 CA VIROGENESIS PLOTS



Contrast	Difference	SE	z ratio	p-value
BTV 10 ATCC - BTV 17 CA	1.15	0.55	2.11	0.10
BTV 10 ATCC - BTV 10&17 CA 50:50	-0.05	0.55	-0.10	0.99
BTV 17 CA - BTV 10&17 CA 50:50	-1.21	0.55	-2.21	0.08

Appendix 3 - BTV-10, BTV-17 CA, and BTV-10:BTV-17 CA 50:50 Infection Groups demonstrate Similar Virogenesis Rates in *C. sonorensis*. BTV-10 (in purple), BTV-17 CA (in blue), and coinfection of BTV-10: BTV-17 CA 50:50 (in green) infection groups demonstrated no statistical difference as determined by pairwise comparison of the linear portion of curve. Each point represents the ΔCt of pool of *Culicoides* (n=5) collected at the indicated day post infection. The ΔCt is calculated as the difference between mean pan BTV CT and *cox1* Ct. Curves were fit to a 3rd degree polynomial. The solid line indicates the model curve with a 95% confidence band. Standard error, z ratio statistic, and p-value adjusted with Tukey's method are given for each comparison.