A study of biting midge populations and an assessment of Bluetongue virus presence in southern Ontario, with a visual dichotomous key to the North American genera of Ceratopogonidae

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Abstract

Biting midges (Diptera: Ceratopogonidae) are flying insects that feed on blood in order to produce eggs. Due to their bloodfeeding habits, some species act as vectors for viruses, many of which affect ruminant animals. This establishes ceratopogonids as possible threats to livestock farmers, and therefore it is important to understand their distribution and habits. This study focussed on collecting biting midge specimens from localities across Ontario where livestock farms are present, and determining if they were carrying Bluetongue virus (BTV).

Fourteen livestock farms were sampled to assess presence of ceratopogonid vectors. Captured *Culicoides* specimens were subjected to RT-qPCR analysis to test for BTV. The North American vector for BTV (*Culicoides sonorensis*) was collected at multiple localities, constituting the first record of this species in Ontario. Identity of *C. sonorensis* specimens was verified using molecular analysis of three gene regions: CO1, ITS1, and EF1α. Gene sequences for *Culicoides variipennis,* a species easily confused with *C. sonorensis,* were also analyzed. Analysis revealed that EF1α introns differed between the species and may be useful as molecular identifiers. Sequences for all three gene regions were submitted to the National Center for Biotechnology Information gene database.

The Brock University Rothamsted Trap operated during the summer seasons of 2013 to 2017, collecting insects at ~12m above ground on a daily basis. Collected biting midges were identified to genus, and genus tallies were compared with five climate variables using redundancy analysis (RDA) to determine factors that affect the activity of collected genera. Phenological patterns of *Bezzia*, *Culicoides*, and *Probezzia* were compared in detail to temperature values.

A literature search was performed to evaluate progression of BTV and epizootic hemorrhagic disease virus (EHDV) across North America since their initial detections in the 1950s. Records

detailing virus outbreaks were analyzed and maps displaying the chronological progression of BTV and EHDV were created, providing a visual representation of their dispersal patterns.

Finally, a dichotomous key to the Nearctic biting midge genera was constructed and illustrated with high-definition photography to show key characters. This key aids with taxonomic identification of the 35 recognized genera occurring north of Mexico.

Preface

The overall objective of this thesis is to examine the presence of biting midge genera in southern Ontario and assess their capability as vectors for Bluetongue virus in the province. Additionally, I sought to develop tools and methods to enhance taxonomical and molecular identification of North American biting midges. This document is organized into seven chapters and prepared in manuscript format.

Chapter 1 provides a brief literature review of the systematics, biology, and habitats of Ceratopogonidae, and details their capability for disease transmission, which is particularly true of members of the genus *Culicoides*. Viruses which are commonly vectored by these organisms are introduced, with information regarding their worldwide distribution, potential hosts, recorded outbreaks, and host symptoms. There is a particular focus on Bluetongue virus (BTV), which this thesis is largely based upon.

Chapter 2 is an extensive literature compendium, examining documentation and scholarly articles focused on North American outbreaks and recordings of BTV and Epizootic Hemorrhagic Disease virus (EHDV), both of which are very similar to each other and commonly jointly referenced as 'hemorrhagic disease' or 'HD'. Included reports date as far back as the 1950s, the decade in which both diseases were first noted in North America, and articles are examined on a decade-by-decade basis. Maps are also provided as visual representations of viral outbreaks per decade, as well as an overall progression map for each virus to illustrate a hypothetical pattern of distribution from 1950 to present day. Potential methods for the gradual expansion of these two viruses are discussed.

Chapter 3 elaborates upon collection data gathered at sheep farms in southern Ontario in the summer seasons of 2013 and 2014. Insects were collected at various different localities in an attempt to understand the composition of biting midge populations occurring in the vicinity of each farm. Captured biting midges were sorted and those with potential capability as vectors for BTV were subjected to real-time quantitative polymerase chain reaction (RT-qPCR) analysis to assess the presence of virus. Results of biting midge capture and RT-qPCR tests are recorded and discussed.

Chapter 4 elaborates upon a significant finding detailed in Chapter III. *Culicoides sonorensis*, the primary North American vector for both BTV and EHDV, was recorded in Ontario for the first time as a result of insect sampling at sheep farms throughout southern Ontario. The details of specimen capture are discussed, as well as the implications of discovering the presence of this vector organism outside of its previously recorded range. Additionally, species identification is verified via comparison of gene sequences isolated from captured specimens to sequences isolated from colony-reared *C. sonorensis* specimens. Molecular markers to distinguish between *C. sonorensis* and its closely related sister species, *Culicoides variipennis*, are determined by analysis of three different gene regions (cytochrome oxidase subunit 1, internal transcribed spacer 1, and elongation factor 1-alpha).

Chapter 5 is an analysis of biting midges captured by the Brock University Rothamsted Trap, which constantly samples insects from the aerial plankton. Captures are tallied on a weekly basis and compared to weekly averages of weather variables to assess relationships between genus abundance and meteorological factors. Observed phenology of three genera displaying the highest abundance throughout all five sampling periods (*Bezzia*, *Culicoides*, and *Probezzia*) is

analyzed. All gathered genus abundance data and climate data is subjected to a redundancy analysis (RDA) to assess patterns and trends in the local system.

Chapter 6 is a visual dichotomous key to aid in identification of North American biting midges to genus level. This key is fully illustrated with high-definition macrophotography and supported with a glossary of important features and terms to facilitate accurate taxonomical identification via physical characters.

Chapter 7 details a collaboration between myself and SEMEX Inc., a biotechnology company with headquarters in Guelph, ON. Their focus involves the rearing of bulls and worldwide transportation of bull sperm. For this collaboration, insect traps were established at SEMEX Inc. farms throughout Ontario to assess the presence of *Culicoides sonorensis* at their facilities, and to determine actions that may reduce the possibility of BTV infection among their cattle populations.

Chapter 8 concludes the thesis with summarizing remarks detailing the primary goals and findings of each thesis chapter. Potential future topics of biting midge and bluetongue virus research are also provided for consideration.

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I would like to thank the numerous professors, entomologists, and institutions who have offered information and assistance during my project. Dr. Art Borkent, Dr. William Grogan Jr., and Dr. Tim Lysyk have been incredibly helpful with respect to tracking down key Ceratopogonidae literature and resources, as well as with examining specimens to help ascertain their identity. Dr. Lysyk also provided some colony-reared *C. sonorensis* specimens to compare with insects I captured during my field season. Dr. Bradley Sinclair who operates in the Diptera unit at the Canadian National Collection of Insects, Arachnids, and Nematodes in Ottawa, ON, as well as Yvonne Linton, one of many curators at the National Museum of National History, have allowed me to loan numerous specimens for photographical purposes.

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Last, but certainly not least, I thank Meagan Tungate, my amazing and supportive partner who has been there for me throughout this endeavour. She has always provided important advice and ideas, and lent me her ear when I needed to talk. She has continually believed in me without question, and that has been an incredible driving force for me. Thank you so much.

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Chapter 1: Literature Review

1.1 Diptera

Class Insecta is subdivided into multiple orders. Members of the order Diptera (the true flies) are characterized by the presence of one pair of flight wings present on the mesothorax and one pair of modified hind-wings (halteres) used exclusively for balance on the metathorax (Downes and Wirth, 1981). This order is further separated into suborders, including the suborder Nematocera (the long-horned flies). Families within Nematocera are often long-legged and delicate, and possess antennae with 6 or more similar antennal segments, a trait which distinguishes them from other dipteran families. Suborder Nematocera contains some of the most significant biting fly families, including Culicidae (mosquitoes), Simuliidae (black flies), and Ceratopogonidae (predaceous midges or biting midges). Certain genera and species within this suborder are known to vector diseases of medical and veterinary significance, establishing them as important organisms for study and monitoring (Mellor, 2000).

1.2 Ceratopogonidae

1.2.1 Taxonomy and Distribution

The Ceratopogonidae, previously known as Heleidae, are known by many common names including biting midges, punkies, and no-see-ums (Wirth, 1952; Marshall, 2006). Ceratopogonids are also occasionally called sandflies, although this may confuse them with phlebotomine members of the family Psychodidae, which are properly known as such (Mellor *et al*., 2000). Ceratopogonids exhibit a global distribution, excluding Antarctica, and the family consists of nearly 6000 known species worldwide (Borkent, 2009). Thirty-nine genera, containing a total of 603 recorded species, are present in North America (Borkent and Grogan, Jr., 2009). Specimens can be found in many different locations, but tend to be particularly

abundant at or near small bodies of water and moist environments, where eggs are laid (Downes and Wirth, 1981).

1.2.2 Biology

Adult Ceratopogonidae frequently possess 13 antennal flagellomeres, although some genera such as *Leptoconops* Skuse, 1889 possess 11 or 12, depending on gender (Downes and Wirth, 1981). Ceratopogonids do not possess ocelli, and also lack a longitudinal groove on the postnotum (Borkent, 2004). Wings possess a very compact radial vein configuration, and a majority of genera also possess a radial-medial (r-m) crossvein. Some genera such as *Alluaudomyia* Kieffer, 1913 and *Culicoides* Latreille 1809, also display obvious wing patterning. In many genera, sexual dimorphism is observed in traits such as antennal structure, antennal plumosity, and obvious differences in external genitalia. Females of biting species possess well-developed biting-sucking mouthparts in order to take blood meals from other organisms, while those of males and non-biting females are reduced. Depending on genus and species, hosts range from invertebrates such as other insects, to vertebrate organisms such as birds, sheep, goats, cows, and other livestock. Adults are mainly crepuscular, but some species may be observed during the daytime (Downes and Wirth, 1981).

Larval ceratopogonids differ in appearance among subfamilies, but generally possess a head capsule showing sclerotization, toothed mandibular segments, and a “collar”-like structure between the head and thorax. Larvae also lack any functional spiracles and are notably strong swimmers. Pupae are very similar between subfamilies, with a compact body structure, a high number of spiracles, and short prothoracic horns which aid in maintaining position at the surface of a water body or moist habitat (Downes and Wirth, 1981; Borkent, 2004).

1.2.3 Life Cycle

Both sexes may acquire nutrients from nectar-rich flowers, but females of predaceous species will often gather protein meals from preferred hosts to facilitate egg development, although those of some species are autogenous and will lay their first set of eggs without requirement of a blood meal (Borkent, 2004). Mating involves swarm assembly, usually above a landmark, and mating itself occurs in mid-flight (Downes and Wirth, 1981). Eggs are generally laid in moist environments, or where there is fecal or decomposing plant matter (Wagner *et al*., 2008). Larvae emerge after a few days and remain semi- or fully aquatic, feeding on microorganisms or other smaller invertebrates. The larvae progress through four larval instar stages, after which they pupate and remain near the top of the water column by utilizing air bubbles trapped under the wing structures, and pupal horns for respiration (Borkent, 2004; Downes and Wirth, 1981). Upon emergence from the pupal casing and cuticular hardening, mating occurs. After receiving a spermatophore from the male, sperm move into the female spermatheca for future egg fertilization. Females then seek out appropriate hosts from which to obtain a blood meal for egg maturation (Borkent, 2004).

1.2.4 Ceratopogonidae as Disease Vectors

Ceratopogonidae have been discovered to transfer a number of microorganisms, including viruses, nematodes, and protozoans, some of which can inflict diseases of medical and veterinary significance to certain hosts. Such species include members of the genera *Austroconops* Wirth and Lee, 1958, *Culicoides*, *Forcipomyia* Meigen, 1818, and *Leptoconops*; however, *Austroconops* does not occur in the Nearctic region (Borkent, 2004) and will not be

included in this study. Of these, *Culicoides* is the most significant threat as members of the genus are capable of vectoring a number of viruses and diseases (Mellor *et al*., 2000).

1.2.5 Genus *Culicoides*

The first formal description of the genus *Culicoides* was established by French zoologist Pierre André Latreille in 1809. Over 1400 species have been found worldwide except for in the polar regions, Hawaii, New Zealand, and Patagonia, and about 96% of those species are known to take a blood meal from mammal and bird species (Mellor *et al*., 2000). Specimens have been known to breed in a broad variety of moist locations, as well as in animal fecal matter and rotting vegetation (Blanton and Wirth, 1979; Wirth and Hubert, 1989). Eggs are laid in groups on a substrate, and larval emergence occurs within 2-7 days. There are four larval stages, the duration of which differs among species and may also be affected by environmental factors such as the ambient temperature. As a result, larval forms may persist for as little as four days or for multiple weeks (Mellor *et al*., 2000). Temperate species are also known to overwinter in the fourth larval instar (Kettle, 1984). In the pupal stage, the organism may float freely in a water column or attach to nearby materials; the pupal stage is often very short in duration (Mellor *et al*., 2000). Adults are crepuscular, and only females take a blood meal to provide nutrients for egg production. Although most do not intentionally disperse very far from their breeding sites, wind currents have been studied and suggested as major contributors to long-range movement of *Culicoides* specimens (Ducheyne *et al*., 2007; Eagles *et al*., 2012). However, an algorithm constructed by Sedda *et al*. (2012) in relation to European *Culicoides* distribution and BTV outbreaks suggests that it is unlikely that *Culicoides* populations would be displaced further than ~30km, and introduction of *Culicoides* into new regions via wind gusts is likely accomplished by

multiple smaller displacements. Adult lifespan is usually between 10 and 20 days, although some specimens have remained alive for as many as 90 days (Mellor *et al*., 2000).

1.2.6 Process of Infection

Transmission of a disease by an arthropod vector may be accomplished mechanically or biologically. Mechanical transmission involves movement of a microorganism from a source to a host via physical means alone, while biological transmission occurs when a microorganism partially or entirely completes its life cycle within the vector organism (Kuno and Chang, 2005). In the case of ceratopogonids, many diseases are vectored biologically. Generally, a biting midge will ingest a small titer of a virus when it takes a blood meal from a host, such as a bird or sheep. While the microorganism is present in the midgut, it migrates through the mesenteron (midgut) wall and enters the haemocoel in the body lumen of the organism. At this point, the microorganism is able to reproduce and circulate throughout the body, gaining access to other organs and structures. In order to spread further to other hosts and vectors, the microorganism must access an organ which facilitates transmission. In the case of ceratopogonids, as well as many other insect vectors, infection of the salivary gland is required for further transmission of arboviruses and other organisms. Microorganisms move through the wall of the salivary gland and enter the lumen, where further propagation may occur. Once the vector ceratopogonid commences feeding upon a new host, saliva containing the disease microorganism is excreted from the glands and into the blood of the host. The timing and duration of viral movement throughout the insect vector is dependent upon factors such as microorganism replication time and host feeding patterns (Mellor, 2000).

1.3 Bluetongue Virus (BTV)

1.3.1 Historical Information

Bluetongue virus (BTV) was first noted in South African livestock sheep in 1902, where it was described as a malarial catarrhal fever (Tabachnick, 1996; Maclachlan *et al*., 2009) and later found to use resident species of *Culicoides* as a transmission vector (Du Toit, 1944). Bluetongue virus has since been discovered in various other regions, such as Asia, Australia, Europe, North America, and the Middle East, although the serotypes in some of these regions are less harmful and generally subclinical. In North America, bluetongue disease was observed affecting Texan sheep, at which point it was named "soremuzzle". In 1952, bluetongue virus was isolated from sheep displaying symptoms of soremuzzle, and *Culicoides sonorensis* Wirth and Jones, 1957 was later identified as a primary vector within the continent (Tabachnick, 1996).

Bluetongue viruses (Reoviridae; *Orbivirus*) are double-stranded RNA viruses, of which there are now 26 known serotypes worldwide (Tabachnick, 1996; Maan *et al*., 2011). Until recently, five serotypes have been known to occur in North America: BTV-2, -10, -11, -13, and -17 (Osburn, 2004; Walton, 2004). Ten other serotypes have been isolated from the southern United States since 1998 (Maclachlan and Guthrie, 2010). BTV is generally found between latitudes of 40°N and 35°S, although recorded cases have occurred at higher latitudes in Asia, Europe, and North America (Osburn, 2004; Maclachlan, 2011). By using *Culicoides* spp. as vector organisms, BTV frequently infects both wild and domestic ruminant organisms, such as deer, sheep, and cattle. Bluetongue virus may also undergo transfer via venereal methods, but this occurrence is extremely rare. In sheep, obvious symptoms of infection include swelling of the buccal and nasal regions, swelling of the tongue, and haemorrhaging within the mouth and

hoof. Symptoms caused by BTV can further induce vomiting, food aspiration, pneumonia, and death. Cattle may also be infected, although clinical symptoms occur in less than 5% of cases (Tabachnick, 1996; Mellor *et al*., 2008).

1.3.2 Prior BTV Outbreaks Outside of North America

Between 1956 and 1960, a significant outbreak of bluetongue, caused by serotype 10, occurred in Portugal and Spain (Tabachnick, 1996; Wilson and Mellor, 2009). BTV-10 was first recognized in Portugal during July of 1956, after which it became prominent in western Spain nearly a month later. The disease may have been carried into the country by affected wind-borne *Culicoides* vectors from Africa. During this outbreak, approximately 180,000 livestock animals were affected by bluetongue; both sheep and cattle were infected, although infection rates of cattle were very low. Control methods were employed to reduce viral spread, such as the slaughtering of infected livestock and restrictions on animal movement. In addition, a vaccine was quickly developed using four strains of bluetongue virus. As a result, every sheep within the area was vaccinated by spring of 1957 and mortality rates were dramatically reduced. It was later discovered that *Culicoides imicola* Kieffer, 1913, a resident ceratopogonid species of Spain and Portugal, was the primary vector involved in BTV-10 transmission throughout the region. After this initial occurrence, BTV did not cause significant harm in the region again for approximately 40 years, at which point smaller outbreaks of various BTV serotypes were observed on the Balearic Islands and the Iberian Peninsula. These outbreaks happened between 1998 and 2012; however, they were much less disruptive than the initial outbreak of 1956 (Pérez de Diego *et al*., 2013).

In 2006, an outbreak of BTV-8 occurred throughout northern Europe, severely affecting livestock populations within Belgium, France, Germany, Luxembourg, and the Netherlands. Incidents of bluetongue reached as far northward as 53°, the highest-ever known latitude achieved by the virus. During this outbreak event, the *Culicoides* genus was incriminated and monitored using an array of different trapping methods, such as blacklight suction traps, Rothamsted traps and CO_2-baited traps (Meiswinkel *et al*., 2008a). Studies focusing on vector competence suggested that three species were capable of BTV transmission in Europe: *C. dewulfi* Goetghebuer, 1936, *C. obsoletus* (Meigen, 1818), and *C. scoticus* Downes and Kettle, 1952. Important information on these organisms was gathered during the outbreak by the use of a 'snapshot survey' of the Netherlands. For this survey, the country was rasterized into a grid of 110 cells, each 20km x 20 km. For 106 of these cells, a farm was chosen for insect sampling using a light trap to assess the presence of *Culicoides* species. Nine traps were used and moved every day until each cell had been sampled. The Obsoletus complex, containing known BTV vectors *C. obsoletus* and *C. scoticus,* was found to be present at 94% of sampled farms in the Netherlands. *Culicoides dewulfi* has been shown to use horse and cattle feces for completion of the life cycle, exemplifying a heavy association with livestock animals. *C. dewulfi* was also recorded at 71% of the farms involved in the snapshot survey, and all three species showed potential to survive long enough to feed on livestock more than once, facilitating virus spread. It is very likely that these vectors were an important factor in the spread of BTV-8 throughout Europe during the outbreak. It was also noted that *C. imicola*, a known significant vector of BTV in southern Europe, was not found in any of the traps, further suggesting that these three resident species were integral to the spread of bluetongue (Meiswinkel *et al*., 2008b).

1.4 Other Diseases Vectored by Ceratopogonidae

African horse sickness virus (AHSV; Reoviridae: *Orbivirus*) is a significant disease affecting horses, donkeys, zebras and other related animals. It is closely related to BTV and was previously thought to be restricted to the continent of Africa; however, several outbreaks have occurred in other regions. The first significant outbreak occurred in Saudi Arabia in 1959, during which the disease spread directly into Iran, Afghanistan, and Pakistan, and likely followed the path of trade routes and major rivers into Cyprus, India, Iraq, Jordon, Lebanon, Syria, and Turkey. Due in part to a mass vaccination attempt, the disease was controlled and infections ceased in 1961. Small outbreaks would continue to arise in various countries and regions within Europe and Asia; however, the virus is currently unable to survive for more than a few years outside of its enzootic region and thus lacks the capability to establish itself for long periods of time. There are nine serotypes of AHSV recognized internationally; serotypes AHSV-4 and -9 were incriminated in the aforementioned outbreak events (Mellor, 1993; Mellor, 1994). Members of the ceratopogonid genus *Culicoides* have been established as transmission vectors for AHSV, as well as some mosquito and tick species, although their potential for transmission of the virus is relatively low (Wilson *et al*., 2009). Infected equines tend to develop circulatory and respiratory complications, as well as hemorrhaging; death may also occur (Mellor, 2000).

Epizootic hemorrhagic disease virus (EHDV) is very closely-related to BTV, belonging in the same family and genus (Reoviridae: *Orbivirus*) and exhibiting very similar properties and structure (Savini *et al*., 2011). Three North American serotypes have been known to inflict sickness and symptoms among livestock: EHDV-1, -2, and -6 (Nol *et al*., 2010; Savini *et al*., 2011). North American incidents of EHDV have been observed between approximately 35°S and 50°N, with outbreaks occurring as far north as southwest Alberta (Savini *et al*., 2011; Pybus *et*

al., 2014). Transmission of EHDV occurs via *Culicoides sonorensis* in North America, and the disease has been known to affect both domestic and wild ungulates, such as cattle and white-tailed deer (Maclachlan and Guthrie, 2010). Sheep and goats have also displayed signs of infection, but these instances were mainly subclinical. Clinical signs may include symptoms such as fever, respiratory difficulties, and swelling of the tongue, amongst other various effects. Death may also occur, although mortality rates differ widely between host organisms (Savini *et al*., 2011).

Leishmaniasis is a disease caused by protozoan parasites of the genus *Leishmania,* which have a worldwide distribution and traditionally exhibit vector-borne transmission by sand flies (Diptera: Psychodidae: Phlebotominae; Seblova *et al.*, 2012). However, some recent studies have focused on ceratopogonid genera as possible vectors for *Leishmania*. Dougall *et al*. (2011) discovered that Australian members of the genus *Forcipomyia* were able to maintain promastigote forms of *Leishmania* parasites within the gut, and their observations also suggest that the parasite could become transmissible within this host genus. Another study performed in the United Kingdom examined the ability of *Culicoides nubeculosus* (Meigen, 1830) to act as a *Leishmania* vector. Parasites were able to develop within the midgut of *C. nubeculosus* prior to gut loading with blood and promastigote *Leishmania*. However, parasite abundance within the gut decreased sharply after defecation of the blood meal, suggesting that parasites were expelled during defecation (Seblova *et al*., 2012). This does not occur in traditional vectors, as the parasites are able to attach to the gut wall of Psychodidae and survive this process (Sacks and Kamhawi, 2001).

Schmallenberg virus (SBV) is an Orthobunyavirus discovered in Europe in 2011, which can cause diarrhea, fever, and abortions in afflicted organisms such as cattle and goats (De

Regge *et al*., 2012). With the recent Bluetongue outbreak of northern Europe in 2006, ceratopogonids have been strongly suspected as vectors for SBV as well, leading to studies focusing on transmission competence of *Culicoides*. Using RT-PCR techniques on the head structures of *Culicoides* species, it was found that SBV was strongly associated with *C. chiopterus*, *C. dewulfi*, and the *C. obsoletus* complex (De Regge *et al*., 2012). Two of these species (*C. dewulfi* and *C. obsoletus*) were also previously implicated as vectors of Bluetongue virus. Schmallenberg virus is not currently an immediate concern for North America, although its discovery demonstrates that viruses which utilize ceratopogonids as disease vectors are still being found, and could potentially become a concern if proper conditions are met.

Vesicular stomatitis virus (Rhabdoviridae: *Vesiculovirus;* VSV) is another microorganism able to be transmitted by *Culicoides* species, such as *C. sonorensis* (Drolet *et al*., 2005; Pérez de León and Tabachnick, 2006). It is known to infect various livestock animals such as cattle, horses, and pigs (Drolet *et al*., 2005; Bennett *et al*., 2008; Pérez de León and Tabachnick, 2006). After initial introduction to a group of animals, VSV is able to spread between susceptible hosts without the aid of an insect vector (Hanson, 1981). Two strains of VSV have caused significant epizootics in the northern United States: vesicular stomatitis Indiana virus (VSIV) and vesicular stomatitis New Jersey virus (VSNJV; Letchworth *et al*., 1999; Pérez de León *et al*., 2006). Infected animals tend to develop a fever, as well as distinctive lesions on structures such as the gums, lips, tongue, and mammaries, and also around the point of infection. In horses, separation of the hoof may occur, leading to further consequences such as deformity or loss of the structure. Strains of VSV may infect humans as well, inflicting a variety of symptoms which last 3-6 days; however, death is not generally a concern (Letchworth *et al*., 1999).

1.5 Trapping Methods for Adult Ceratopogonidae

Ceratopogonid specimens can be collected using many different methods. However, the primary methods of collection for this project will be via carbon dioxide (CO_2)-baited light traps and the Brock University Rothamsted trap on Decew Road in Saint Catharines, Ontario.

The first Rothamsted suction trap was constructed in 1964 in the town of Rothamsted, United Kingdom (Barbagallo, 1982). Additional traps were later erected in the UK as well as throughout eastern and western Europe, allowing insect populations to be monitored in multiple areas (Fassotte *et al*., 2008). The design of the trap consists of a 9.2-metre tall pipe-shaped column, with a 3-metre tall box at the base containing an accessible sample container, collection net, and an electric fan to create suction. Insect specimens are pulled downward toward the base of the column, where they enter the collection vessel and are killed (Macaulay *et al*., 1988). Rothamsted traps are always operational, constantly sampling the insects that pass overhead at a 12-metre elevation from the ground. This specific elevation was selected for trap construction because it excludes locally-flying insects, but is also near enough to the ground that it can effectively sample migrant insects (Macauley *et al*., 1988; Taylor and Palmer, 1972). This simplifies the capture of crepuscular insects such as ceratopogonids, and provides an unbiased subsample of organisms in the aerial plankton at 12m above ground. In the UK, Rothamsted traps were previously employed for the monitoring of pest organisms such as aphids, but were also later utilized for the monitoring of *Culicoides* vectors throughout Europe during the BTV outbreak of 2006 (Macauley *et al*., 1988; Fassotte *et al*., 2008).

CDC miniature light traps, constructed by the John W. Hock Company (Gainesville, FL), may also be used to capture *Culicoides* specimens. General trap structure consists of a small circuit operating an ultraviolet light and a small rotary fan positioned just below the bulb. As

insects are attracted to the light source, they are drawn through the fan via suction and pushed further downward into a ventilated collection vessel. This basic mechanism is shielded from above by a round, flat "saucer" structure which protects the circuitry, mechanisms, and specimens from unfavourable weather such as rain. The mechanism is often powered by a large 6-volt battery, which must be recharged after use. Additionally, the trap may be outfitted with a dry ice container overtop of the mechanism, which distributes carbon dioxide for use as a biting insect attractant (John Hock Company, 2009).

Carbon dioxide is frequently used to capture biting insects such as mosquitoes, but studies have demonstrated its usefulness for the collection of ceratopogonid specimens as well. Carbon dioxide-baited traps were used to monitor *Culicoides* during the BTV outbreak in Europe, 2006 (Meiswinkel *et al*., 2008a). Grant and Kline (2003) performed a study in which *Culicoides* reaction to CO_2 levels was examined. They found that *Culicoides furens* (Poey, 1853) showed an electrophysiological response to levels as low as 150 ppm; two other species, *C. stellifer* Coquillett, 1901 and *C. mississippiensis* Hoffman, 1926, also showed a similar and comparable reaction. It is postulated that this may allow for better detection not only of vertebrate hosts, but also of nectar-rich regions of plants for sugar-feeding which employ volatile compounds in conjunction with carbon dioxide. However, it may also be that some insects are adapted to lower levels of CO_2 from their existence prior to heavy industrialization, which could also be the case for *Culicoides*. Another study was published in 2012 which focused on effectiveness of carbon dioxide paired with 1-octen-3-ol as an attractant method for *Culicoides* specimens. The study found that traps baited with both CO_2 and octenol were more successful than those baited with only CO_2, demonstrating that octenol may have significant potential as a complimentary additive to carbon dioxide trapping methods (Harrup *et al*., 2012).

Trapping methods that use ultraviolet light as an attractant may be used to capture many different types of insects, including Nematoceran biting flies such as ceratopogonids (Gutsevich and Glukhova, 1970; Iwakama and Reeves, 2011; Venter *et al.*, 2012). During the European outbreak of 2006, Onderstepoort black-light traps were used to great effect for gathering collection data from farms for the snapshot survey used to monitor *Culicoides* bluetongue vectors (Meiswinkel *et al*., 2008b). A study performed by Venter *et al*. (2012) examined the efficacy of Onderstepoort light traps for capturing *Culicoides* midges in South Africa. The authors found that the trap was only effective in a limited range around the point of trap placement and would not likely exceed a maximum coverage area of 50 m^2. Additionally, the types and abundances of species captured by one Onderstepoort trap was likely to be dependent upon the environment the trap was set in, as well as ambient conditions during trap operation time. Despite these factors however, Venter *et al*. suggested that Onderstepoort traps are still quite useful for monitoring specific areas in the correct environmental conditions and timeframe. Centre for Disease Control (CDC) light traps are also frequently used for insect monitoring within North America, which use ultraviolet light and optional carbon dioxide as attractants. Recent surveys of *Culicoides* in Alberta cattle facilities and South Carolina zoos utilized CDC black-light traps to capture specimens, resulting in the successful attainment of a variety of species (Lysyk, 2006; Nelder *et al*., 2007).

Live animal-baited traps are another alternative for collection of biting insects. These traps often utilize a stationary livestock animal positioned underneath a net, which captures any biting insects that are attracted to the animal. A form of live animal trap was utilized by Harrup *et al*. (2012) for their study on the efficacy of 1-octen-3-ol as an attractant. In their study, a live-baited drop trap as described in Carpenter *et al*.'s experiment (2008) was used to capture

Culicoides specimens for a survey in Scotland. Results showed that the live baited drop trap was able to collect over three times as many *Culicoides* specimens when compared to an octenol-baited trap (Harrup *et al*., 2012). This demonstrates that the use of live hosts for the capture of ceratopogonid specimens, alongside other trapping methods, may be beneficial.

1.6 Molecular Methods and Techniques

Polymerase chain reaction (PCR), a molecular method developed by Kary B. Mullis in 1983, is a process by which specific targeted DNA sequences can be replicated and amplified (Mullis, 1990). This simplifies detection of the targeted sequences by increasing their presence in a DNA mixture. There are three main stages to PCR. Firstly, double-stranded DNA is converted to single-stranded DNA by subjecting it to heat and melting the genetic material. Secondly, primers adhere to areas on the DNA sequences which flank the targeted gene sequence. Finally, DNA polymerase induces extension of the primers across the target sequence, which duplicates the material. To facilitate these reactions, the material is often placed into a thermal cycler, which maintains it at the required temperatures for each portion of the process (Steffan and Atlas, 1991).

In the case of some insects, such as *Culicoides*, determination by the use of morphological characteristics can be extremely difficult. In these instances, the results of PCR analysis may be used to distinguish closely-related species of organisms based on differing genetic information, establishing the process as a useful tool for insect identification and phylogeny construction. Genetic sequences frequently used for PCR analysis of insects are the mitochondrial cytochrome oxidase subunit I and II (CO1 and CO2) genes (Guryev *et al.*, 2001; Beckenbach and Borkent, 2003). The use of these specific gene sequences is beneficial for a

variety of reasons. Firstly, there has been a large amount of research previously performed using the CO1 and CO2 sequences in a number of insect groups, since they are regularly used for the construction of phylogenetic trees within Insecta (Caterino and Sperling, 1999; Dobler and Müller, 2000; Guryev *et al*, 2001). Secondly, the CO2 sequences of closely-related families such as the blackflies (Simuliidae) and non-biting midges (Chironomidae) are available, which can be used for comparison to different but phylogenetically-close groups (Beckenbach and Borkent, 2003).

Real-time Quantitative Polymerase Chain Reaction (RT-qPCR) is a variation on the standard PCR method that allows for quick visualization of results. This is accomplished by introducing a fluorescent marker into the PCR solution, which attaches alongside the targeted gene sequences. As PCR progresses, the marker fluoresces as a result of being cleaved from the activity of DNA polymerase. The change in fluorescence can then be measured, and the presence of targeted DNA sequences can be estimated from this observation (Saijo *et al*., 2008). This method of analysis can be used for the identification of insects such as biting midges, as exemplified by a study performed by Monaco *et al*. (2010) in which three species within the *Culicoides obsoletus* species complex were successfully discerned from one another using real-time PCR. Results were compared with those achieved using standard PCR, showing complete agreement between the two processes. Another study performed on Swiss species of *Culicoides* found that RT-qPCR was effective at identifying a single midge (*C. imicola*) from a pool of 100 ceratopogonid specimens, but produced some incorrect false-positive results in pools with a higher number of specimens (Wenk *et al*., 2011). This illustrates that RT-qPCR is a sensitive process which can be a useful tool for quick determination of species; however, it is not without limitations and must be used judiciously.

Reverse Transcription/Transcriptase Polymerase Chain Reaction (RT-PCR) is frequently confused with real-time PCR; however, it is an entirely different molecular process by which RNA can be quantified. With the introduction of reverse transcriptase to an RNA sample, the RNA is used to produce complementary single-stranded DNA. After this reaction, the DNA can be amplified by the use of standard or real-time PCR (Freeman *et al*., 1999). Many diseases vectored by ceratopogonids and other biting nematoceran insects are RNA viruses, including bluetongue virus (Mellor, 2000). Therefore, if a ceratopogonid specimen is harbouring an arbovirus or other such organism, RT-PCR can be utilized to amplify the genetic material of the virus and demonstrate its presence or absence.

1.7 Dichotomous Keys

One of the most widely-used methods for taxonomical identification is the dichotomous key, which can separate organisms at varying taxonomical levels based on taxon-specific characters. Although these keys are mostly based upon morphological traits, some may also make use of other discerning qualities such as distributional information and behavioural observations. Dichotomous keys were primarily used for identification of plantlife, but they have since been used for identification of animals, fungi, and minerals, amongst other natural organisms and materials. The concept of identification by comparison of traits between organisms has been traced as far back as 1689, when British botanist Richard Waller proposed a method of botanical identification based on comparison of plant specimens to watercolour images, detailing their physical characteristics (Griffing, 2011).

Dichotomous keys are structured as a series of couplets, with a couplet containing two descriptors for a particular trait. These descriptors are compared to the organism or compound

under study, and the identifier is to discern which descriptor best pertains to the item or organism they are examining. Upon selection, the couplet will either refer the user to another couplet located elsewhere in the key, or will provide identification information, if it has been reached. This process is repeated until the object of study is identified by reaching a couplet that no longer leads to a subsequent couplet and provides identification information instead, such as a taxonomical identifier. Couplets may include numerous characters for comparison and may also refer to diagrams to properly illustrate the characters under scrutiny to assist identification. Recent dichotomous keys have begun to incorporate high-definition photography of characters from collected specimens to best exemplify key identifiers. Additionally, many dichotomous keys are made available online for public access and may be made interactive on computer systems for ease of use. For example, the Canadian Journal of Arthropod Identification has published 33 documents (as of the development of this thesis) with visual and interactive dichotomous keys for the identification of various arthropod taxa (Canadian Journal of Arthropod Identification, 2018).

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Chapter 2: Geographical Expansion of Bluetongue Virus (BTV) and Epizootic Hemorrhagic Disease Virus (EHDV) in North America (1950-present)

2.1 Introduction

Bluetongue virus (BTV) is a debilitating *Orbivirus* disease (family Reoviridae) that can affect a number of ruminants, including important livestock such as sheep, cattle, and goats. Originally discovered over a century ago in European livestock that had been transferred to Africa, BTV has since been isolated in a number of other regions, causing numerous severe outbreaks and widespread livestock death. The first record of BTV in North America was in California in 1952 when a sheep tested positive for serotype 10 (BTV-10; Tabachnick, 1996). Since this initial finding, four other serotypes have commonly been isolated across the continent (BTV-2, -11, -13, and -17; Walton, 2004) and are considered to be endemic. There are currently 26 recorded BTV serotypes worldwide (Maan *et al*., 2011); however, many of the non-endemic serotypes have been recorded in North America without becoming permanently established (Barber *et al*., 2010). Symptoms of BTV are often observed in goats, deer and sheep, although clinical indications only arise in a small fraction of infected animals; many infections are subclinical and go unnoticed. Clinical symptoms that may develop, however, include swelling and hemorrhaging of the nasal and buccal regions, cyanosis of the tongue, esophageal and pharyngeal lesions, excessive salivation, increased body temperature, and hemorrhaging in the hooves. Death may occur when vomit, mucous, and other fluids enter the lungs. Cattle that suffer from infection rarely become symptomatic, and symptoms are much less severe if they develop (Tabachnick, 1996). It has been suggested, however, that cattle function as reservoirs for BTV, allowing it to persist in livestock populations for extended periods of time (Walton, 2004).

Epizootic hemorrhagic disease virus (EHDV) is another debilitating ruminant virus classified in the same family and genus as BTV. EHDV was originally described in 1955 from a white-tailed deer in New Jersey, USA, but has since become widespread throughout the United

States, with a few rare occurrences in western Canada. Although 8 serotypes of EHDV are currently recognized, it has been suggested that some serotypes are identical to one another and may be amalgamated in the near future (Savini *et al*., 2011). Upon introduction to the host, EHDV initially replicates within the local lymphatic structures near the site of infection. Following this, the virus enters the bloodstream where it travels to additional lymph nodes and eventually enters the spleen. There are several different symptoms classified under three clinical forms: per-acute, acute, and chronic. During per-acute EHDV, the host often exhibits edema of the cranial region, fever, swelling of the tongue, respiratory complications, and anorexia; this is often followed by rapid death, occurring within 36 hours. Acute clinical signs include per-acute symptoms, as well as tissue hemorrhaging, salivation, excessive mucus production, and lesions in the buccal and nasal areas. Chronic symptoms occur when the host becomes ill but recovers; clinical signs include damage to the stomach rumen, lesions throughout the body, and hoof and toe damage that may prompt animals to crawl on their knees (Savini *et al*., 2011).

New viral serotypes continue to be found worldwide, and are frequently observed in areas outside of their known range. Recent trends in meteorological data have shown gradual increases in temperature throughout North America, resulting in milder winters with later initial snowfalls, and earlier snowmelt in the spring in some areas (Environment and Climate Change Canada, 2013). Additionally, temperature ranges of more northern latitudes have increased, allowing for species and diseases that regularly exist in more southern regions to expand into previously uninhabitable areas, potentially leading to permanent establishment. Through expansion of insect species, for example, new vectors for disease may become present and possibly bring new microorganisms with them. Increased temperature may also influence viral persistence in new regions, which may allow a previously unrecognized vector to acquire it. Therefore, significant

impacts upon local animal populations are a possibility as harmful microorganisms are introduced to them.

The most common transmission organisms for BTV and EHDV throughout the world are members of the genus *Culicoides* Latreille, 1809, belonging to the insect family Ceratopogonidae (biting midges; Carpenter *et al*., 2015). In North America, the most efficient vector for BTV and EHDV is currently the species *Culicoides sonorensis* Wirth and Jones, 1957. This species has been recorded in many of the United States, and has also been collected in the Canadian provinces of British Columbia, Alberta, and recently, Ontario (Shapiro *et al*., 1991, Jewiss-Gaines *et al*., 2017). Two years following the discovery of *C. sonorensis* in Ontario, BTV-13 seropositivity was detected in three cattle from Ontario's Chatham-Kent region in August 2015 (OIE, 2015). These animals were born and raised locally, ruling out the possibility of viral import during transportation of the host into the region, and suggesting that BTV has recently been introduced to Ontario in some way. In the report, it is noted that one of the three cattle was slaughtered in an effort to contain the virus. There is a distinct risk that the virus has become established in the province rather than existing as a temporary introduction, and could persist, expand its range, and begin to affect more branches of the Ontario livestock industry.

It is important to document and analyze the spread of potential disease vectors as well as the diseases themselves to evaluate expansion patterns and establish plans for awareness, prevention, and treatment as early as possible. This study will examine previous peer-reviewed and gray-area literature (articles, reports, news items) for information regarding bluetongue virus, as well as the closely-related epizootic hemorrhagic disease virus (EHDV), which often cross-reacts with BTV serological tests; due to their high degree of similarity, BTV and EHDV are often lumped together and collectively referred to as “Hemorrhagic Disease”, or HD. The

goal of this review is to provide timelines of the introduction and proliferation of BTV and EHDV throughout their recorded history in North America.

2.2 Materials and Methods

Primary journal articles, as well as articles, news items, or government reports detailing evidence of BTV, EHDV, or "HD" (the umbrella term for both diseases) were selected for any information pertaining to outbreaks or notable infections. Articles were included if they provided evidence of viral isolation, seropositivity in infected animals, or physical symptoms of disease.

This review is organized by decade, beginning with 1950-1959 and ending with 2010-present. Articles were included based on the date that their studies took place, rather than the date of publication. In some cases, research articles or other pieces of information spanned multiple years. These resources were included in the decade range in which their primary research was concluded. For example, a study conducted between 1998 and 2004 would be included in the 2000-2010 section. For each decade, distribution maps for BTV and EHDV occurrence were created using ArcMAP 10.5.1, with the diseases highlighted in various shades of blue and red, respectively. Maps display provinces and states in North America (excluding Mexico) that exhibited new records of virus, had previous record of virus, or had previous record of virus with a reoccurrence during the decade being discussed; states or provinces with no record of virus are white. Two additional maps were constructed to summarize history of new BTV and EHDV records by decade as they were recorded throughout the continent, resulting in a timeline of viral reports from January 1950 to present day.

2.3 Results

2.3.1 BTV Prior to North American Discovery

Bluetongue virus was initially implicated in livestock sickness in South Africa prior to the 1900s, and is likely to have occurred there ever since sheep farming was established in the country (Henning, 1956). From these incidents, the effects upon livestock were described as a livestock fever that occurred mainly during the summer and was particularly harmful in seasons with high amounts of precipitation. From these occurrences, the first description of 'malarial catarrhal fever' was penned by Hutcheon and Spreull in the early 1900s, after which the current name of Bluetongue was established by Spreull based upon the Afrikaans name for the disease, 'Blaauwtong'. Hutcheon and Spreull also first suspected that the disease was transmitted by an insect vector in 1905, and suggested that it persisted in environmental conditions similar to those that favour African horse sickness virus. The disease-causing agent was first identified as a virus by Sir Arnold Theiler in 1906, who also developed the first vaccine for BTV (Henning, 1956). Eventually, it was theorized that a type of biting insect was responsible for bluetongue transmission, leading to identification of *Culicoides* biting midges as primary vectors by Rene du Toit (1944). Before its discovery in the United States, as well as numerous other countries across the globe, BTV was presumed to be completely restricted to Africa, Israel, and a few Mediterranean regions, including Cyprus (Maclachlan, 2011). However, it has since become established in a vast number of regions located between roughly 40°S and 35°N, with some recorded incidents at even more northerly or southerly latitudes (Maclachlan, 2011).

2.3.2 Timeline of BTV and EHDV Recordings in North America

2.3.2.1 1950-1959: Initial Realization that BTV and EHDV were Present in North America

Although the first instance of bluetongue was merely considered infection by a bluetongue-like illness in the late 1940s, the initial observation and identification of BTV in North America occurred in the state of California, 1952, where it was isolated from a flock of sheep appearing to show symptoms of a disease previously known as 'soremuzzle' (Tabachnick, 1996). Additional reports suggest that it was also present in various regions of western Texas in the same year, with descriptions of affected sheep and warnings that soremuzzle differed from a superficially similar livestock disease called contagious ecthyma, or 'sore mouth' (Society for Science & the Public, 1952). This BTV serotype was identified as BTV-10 (Maclachlan, 2008).

Shortly after the discovery of BTV, the closely-related epizootic hemorrhagic disease virus caused an outbreak in the United States. An estimated 500-700 white-tailed deer were killed by an unknown disease in New Jersey during the late summer of 1955. It was noted that many dead specimens were located near water sources, a typical observation that occurs in cases of both EHDV and BTV. EHDV-1 was isolated from a white-tailed deer in the region, marking the first EHDV outbreak in the country. Similar deaths of white-tailed deer were reported from Michigan and North Dakota in the same season, suggesting that EHDV-1 was the cause of these outbreaks as well (Hecht, 2010).

Cattle-borne bluetongue was originally isolated from livestock in Jackson county of Oregon, 1959, which exhibited clinical symptoms initially recognized as 'mycotic stomatitis'. Blood was drawn from cattle displaying signs of disease and injected into test sheep housed at the Arthropod-Borne Animal Disease Research Laboratory in Denver, Colorado, inducing symptoms in the infected sheep (Hourrigan and Klingsporn, 1975). In addition, Hourrigan and

Klingsporn (1975) provided a detailed timeline of BTV discovery throughout the United States between 1955 and 1973, which presents information regarding initial isolations of the virus in various US states (Table 2.1).

Table 2.1. Year of initial recording of Bluetongue virus isolation in 17 states, as reported by Hourrigan and Klingsporn (1975).

US State	Year of Initial BTV Isolation
Texas	1952
Arizona	1954
Colorado	1954
Kansas	1954
Nebraska	1954
Missouri	1955
New Mexico	1955
Oklahoma	1956
Oregon	1956
Utah	1956
Nevada	1957
Idaho	1958
Montana	1962
South Dakota	1962
Wyoming	1962
Washington	1966
Indiana	1967

A brief BTV epizootiology report by Walton *et al*. (1984) discussed the distribution of BTV serotypes throughout North America from the 1950s to the early 1980s. They reported that various serotypes had been isolated from mammals or insects in 32 of the United States since BTV's initial isolation in California, 1952. In addition to the aforementioned isolation of BTV-10 in California, the authors mentioned isolation of BTV-11 in New Mexico, 1955, BTV-17 in Wyoming, 1962, and BTV-13 in Idaho, 1967. Additionally, they stated that serological studies and surveys had discovered infected animals in every state except for Alaska and Rhode Island,

although they noted that this wasn't an accurate depiction of presence of actual virus in those states due to transportation and trading of animals throughout the country.

It is very briefly mentioned in a 2014 report that an outbreak of BTV occurred in Washington State sometime in 1959 (Noga, 2014). Explicit details are not provided, and the document itself states that no serotype identity was confirmed. This particular outbreak does not appear to be substantiated in other literature sources reporting BTV occurrences at the time, and so it is likely not accurate. This inaccuracy is further corroborated by Hourrigan and Klingsporn (1975), who state that the first isolation of BTV in Washington State did not occur until seven years later in 1966.

Maps displaying BTV and EHDV occurrences from 1950 to 1959 can be seen in Figures 2.1 and 2.2.

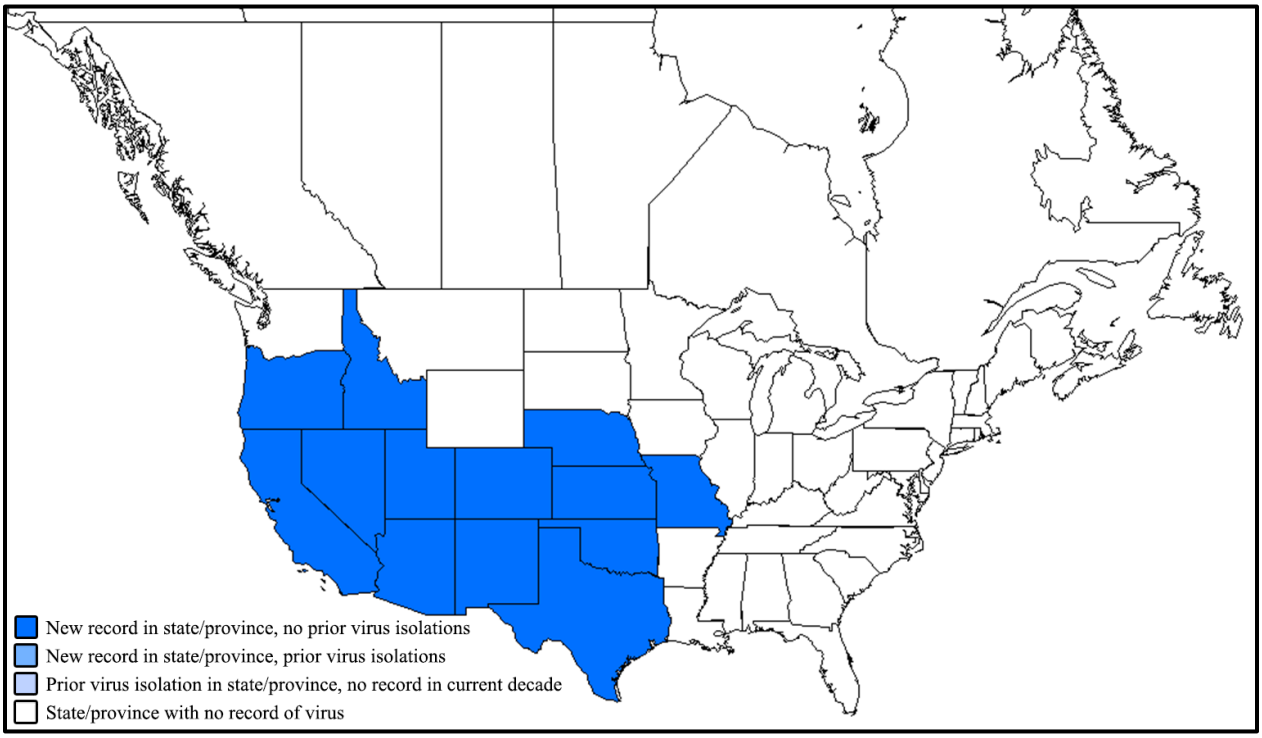

Figure 2.1. State and provincial records of BTV incidents in North America from January 1st, 1950 to December 31st, 1959.

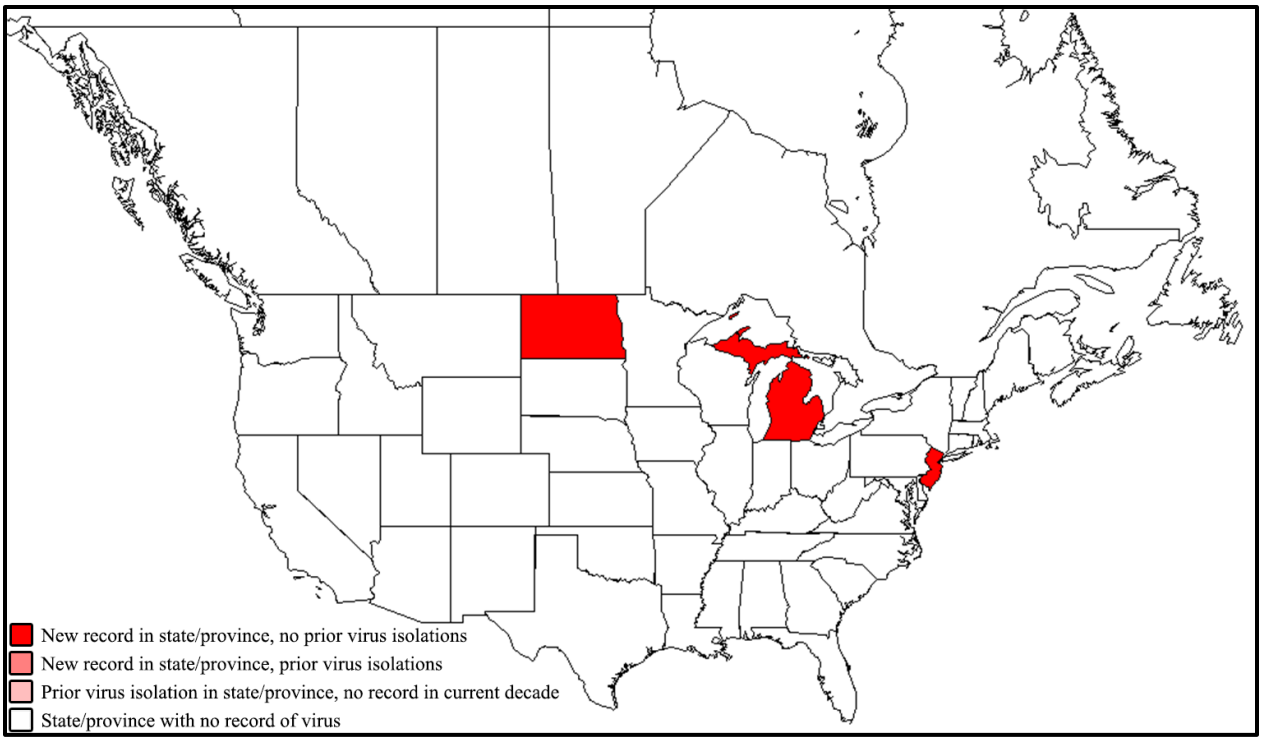

Figure 2.2. State and provincial records of EHDV incidents in North America from January 1st, 1950 to December 31st, 1959.

2.3.2.2 1960-1969: Northward Expansion of BTV and EHDV

According to Hourrigan and Klingsporn (1975), a number of US states saw first isolations of BTV in the early 1960s, including Indiana, Montana, South Dakota, Washington, and Wyoming (Table 2.1). Unfortunately, they do not provide detailed accounts regarding how these isolations came about, but it is mentioned that these isolates were made from both sheep and cattle in numerous counties from each state; their data were provided by the US Department of Agriculture.

In 1962, EHDV was found to be responsible for sickness and death in wild ruminants throughout southwest Alberta. Molecular analysis found this particular serotype to be EHDV-2, which has since been considered endemic to the area (Allison *et al*., 2010). During this outbreak,

it was noted that over 450 white-tailed deer, as well as a number of mule deer and pronghorn antelope, were discovered dead as a result of EHDV infection. It is suspected that outbreaks in this southern region of Alberta often coincide with outbreaks noted in the northernmost United States. This relationship is attributed to wind currents that move northward, carrying infected *Culicoides* with them and introducing non-infected *Culicoides* that are capable of acquiring the virus from local sources (Pybus *et al*., 2014).

Maps displaying BTV and EHDV occurrences from 1960 to 1969 can be seen in Figures 2.3 and 2.4.

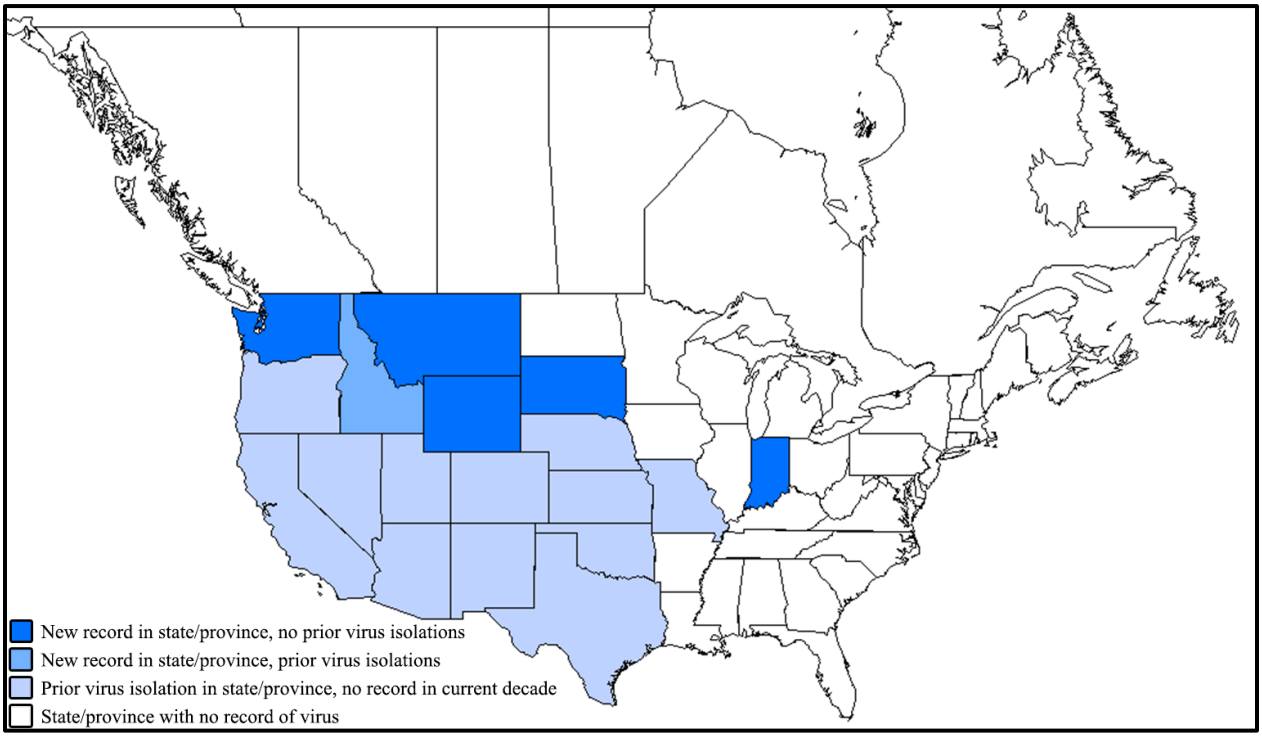

Figure 2.3. State and provincial records of BTV incidents in North America from January 1st, 1960 to December 31st, 1969.

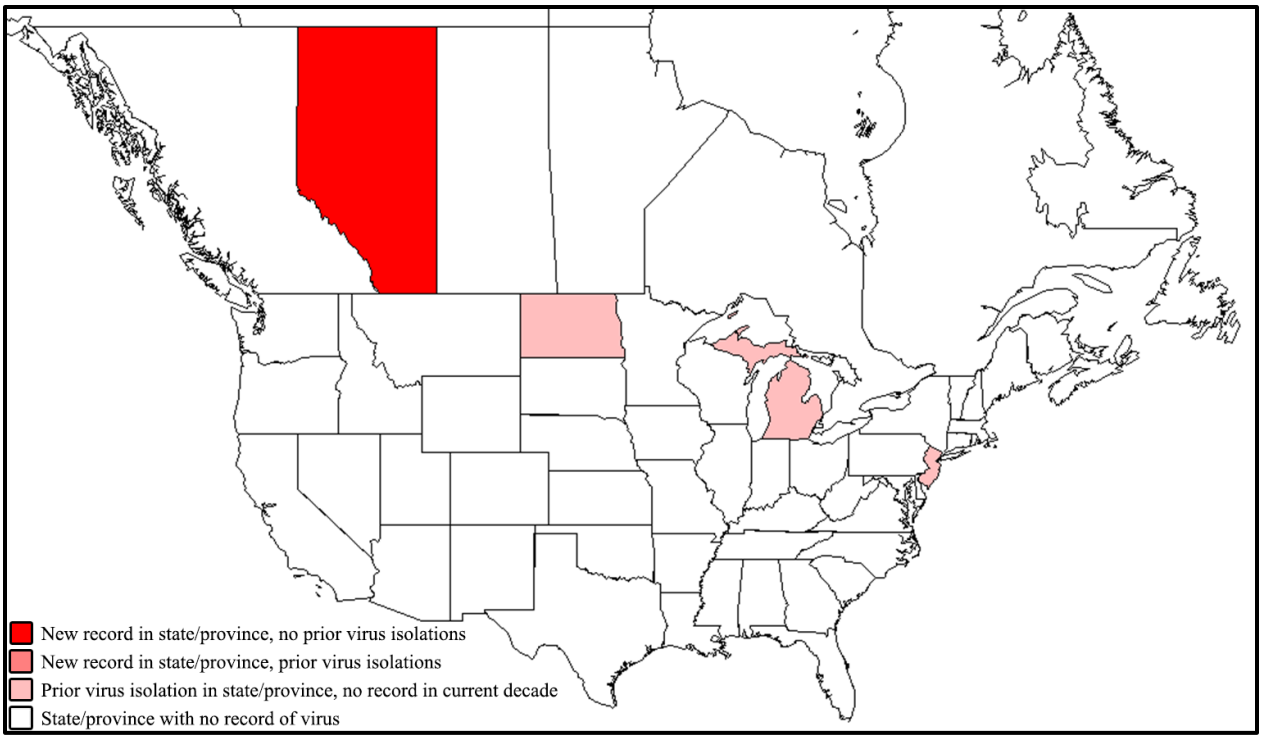

Figure 2.4. State and provincial records of EHDV incidents in North America from January 1st, 1960 to December 31st, 1969.

2.3.2.3 1970-1979: BTV Discovery in Canada and Other Significant Observations

In addition to their prior information, Hourrigan and Klingsporn (1975) include reports of a few BTV incidents in the early 1970s. Specifically, BTV was isolated from cattle in Minnesota, Kentucky and Florida between 1970 and 1973, though detailed information is not provided about these incidents. In addition, some 'bluetongue-like' isolates were extracted from afflicted deer between 1971 and 1972 in Tennessee, North Carolina, and Colorado; however, since these incidents do not expressly indicate if the isolated virus was BTV, or even the closely-related EHDV, they will be discounted for the purpose of this review.

One of the most significant discoveries regarding Bluetongue virus in the 1970s was the first isolation of BTV in Canada. In 1975, 261 livestock cattle, which had been imported to

British Columbia from the United States, were found to be seropositive for BTV and were subsequently destroyed (Thomas *et al*., 1982). Furthermore, 221 cattle that had previously been in contact with the seropositive flock also showed infection when they were tested in 1976. These findings led to establishment of a quarantine zone within British Columbia that included a large portion of the Okanagan Valley bordering Washington State; any seropositive animals detected within this zone were slaughtered to contain the virus. In the following years until 1979, a country-wide survey for BTV was performed for further monitoring and containment, but only 24 infected cattle were found, the majority of which were isolated within, or in areas adjacent to, the established quarantine zone; two of the infected animals were imported from the USA. Additionally, 15 sheep, 7 goats, and 4 deer within the quarantine zone were found to be seropositive during the survey in 1976. This sharp drop in observed infections led to the conclusion that BTV was no longer present in Canada.

Bluetongue virus was also responsible for a sizeable outbreak in the state of Wyoming during 1976 (Thorne *et al*., 1988). In early September, the northern Wyoming counties of Sheridan and Campbell exhibited mortality in pronghorn deer. Only two days after this report, additional pronghorn deer deaths were reported from the county of Niobrara further south, where a symptomatic pronghorn was shot and killed for study. Eleven pronghorn specimens were examined via necropsy during this event, and four additional samples of pronghorn tissue originating from various counties were also analyzed. Examination of blood serum and spleen tissue was performed using modified direct complement fixation (MDCF) testing. BTV-17 was isolated from three pronghorn tissue samples; serum analysis from these specimens also revealed BTV antibodies in the blood. It is noted that additional serum samples were collected from hunter-killed pronghorn (94 specimens) and deer (38) near the end of this outbreak. Analysis

found that 76/94 pronghorn and 36/38 deer displayed evidence of BTV exposure. It is also noted that similar numbers of specimens tested positive for EHDV antibodies, though exact numbers were not provided. Overall the Wyoming Game and Fish Department approximated that over 3,200 pronghorn deer were killed by the virus during this incident, as well as over 1,000 specimens of other deer species (mainly mule deer). Samples from 25 pronghorn and 45 deer were gathered from the same region roughly one year later to re-evaluate presence of BTV. Analysis found that 15 pronghorn specimens had BTV antibodies in their sera, and 12 tested positive for presence of EHDV antibodies. Of the 45 deer samples, 32 and 25 specimens tested positive for BTV and EHDV antibodies, respectively.

A significant outbreak of hemorrhagic disease was also documented in Montana in the mid to late 1970s (Feldner, 1980). In 1976, over 30 localities, mainly situated within the counties of Powder River and McCone, reported numerous deer mortalities in the summer season. This persisted in 1977, with a number of white-tailed deer deaths observed near the Yellowstone River in two more counties, Dawson and Richland. Further deer deaths were reported in 1978 in the southeastern areas of the state. It is estimated that roughly 500 white-tailed deer were killed by HD during this outbreak. Serum and tissue samples from affected deer were sent to a number of laboratories and virologists throughout North America, and members of the Wisconsin Veterinary Science Laboratory determined that BTV, EHDV, or possibly both viruses, were the disease agents involved (T.M. Yulli, personal communication as cited in Feldner, 1980). In 1980, a thesis study was conducted to determine which of these viruses was responsible for the outbreak. By analyzing serum samples from Montana deer and cattle, 73% of mule deer were found to be positive for EHDV, while only a small fraction (5%) of tested white-tailed deer showed EHDV seropositivity; the author suggested that trend was due to the widespread death of

EHDV-infected deer that were infected during the outbreak, limiting the remaining population to those that were uninfected (Feldner, 1980). These findings suggested that EHDV was the causative agent of the outbreak from 1976-1978.

Maps displaying BTV and EHDV occurrences from 1970 to 1979 can be seen in Figures 2.5 and 2.6.

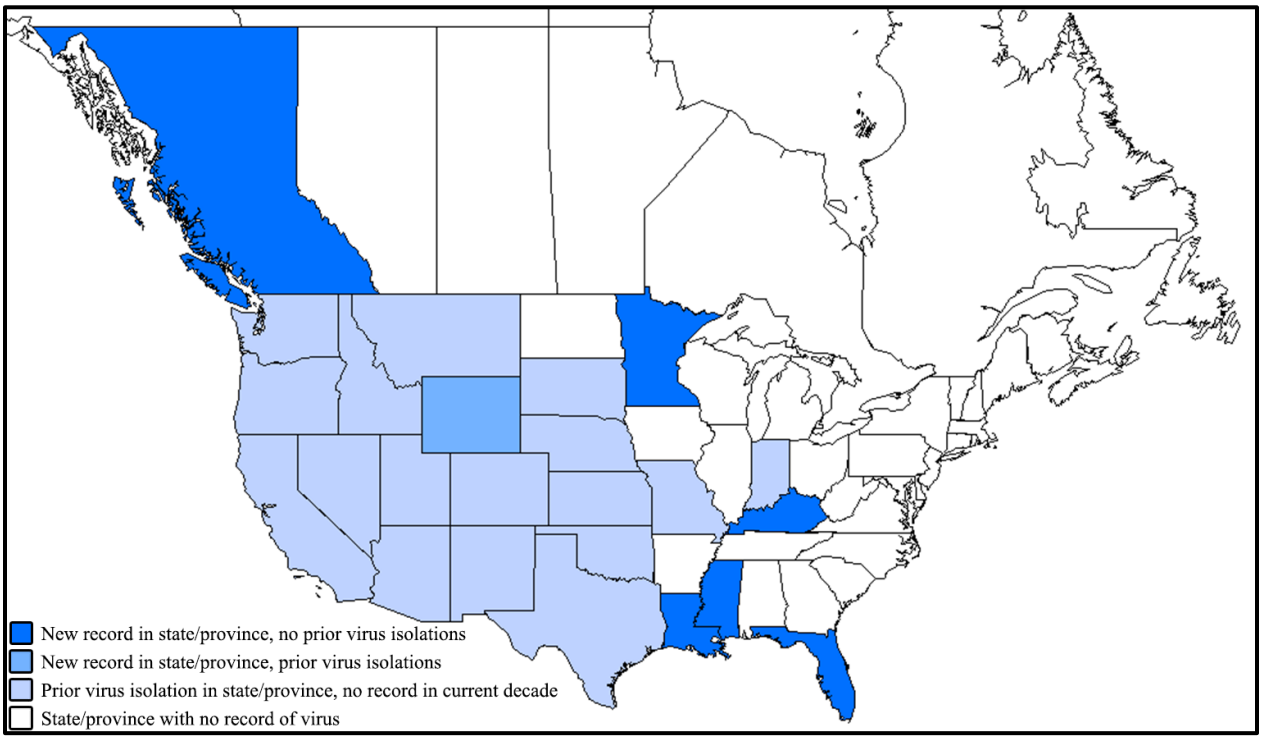

Figure 2.5. State and provincial records of BTV incidents in North America from January 1st, 1970 to December 31st, 1979.

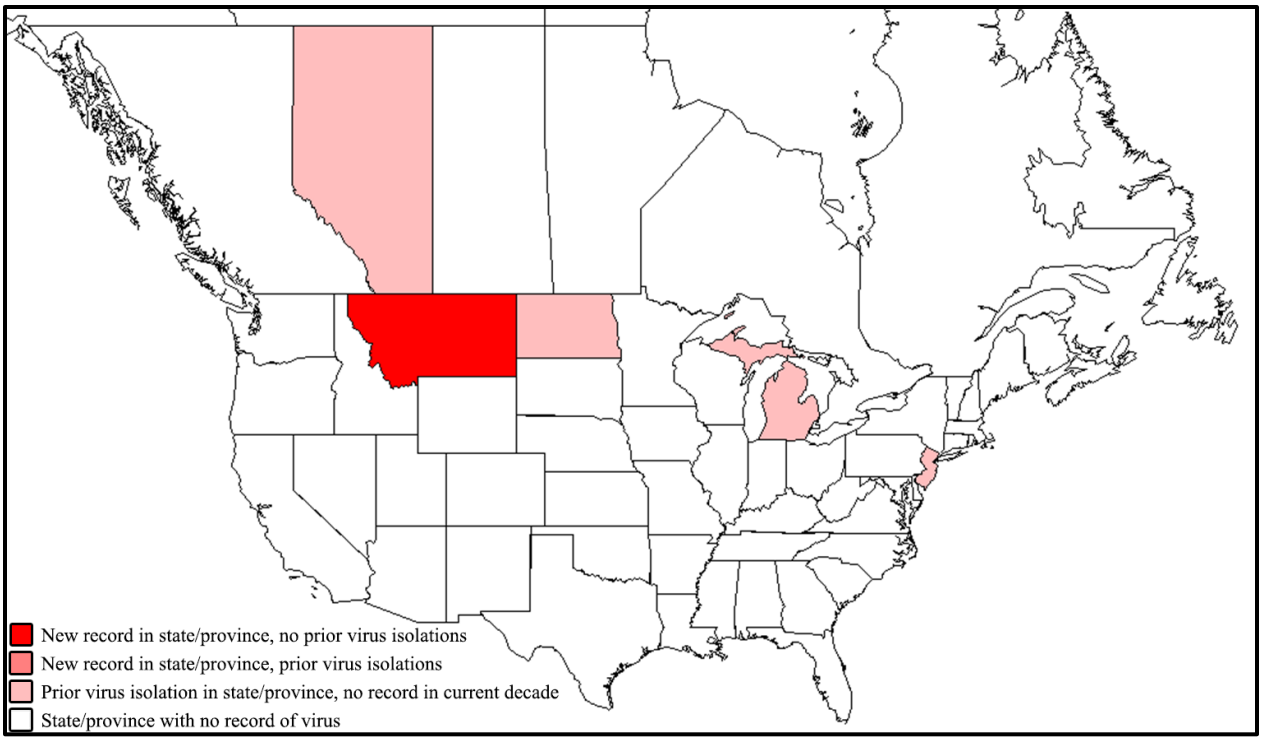

Figure 2.6. State and provincial records of EHDV incidents in North America from January 1st, 1970 to December 31st, 1979.

2.3.2.4 1980-1989: EHDV Discovered in British Columbia

Hugh-Jones *et al*. (1989) examined the presence and distribution of BTV in the state of Louisiana between April 1982 and September 1984. Serum samples had been collected from 274 cattle herds throughout the state as part of a program for brucellosis control and eradication. From each herd, a maximum of 100 cattle were sampled, resulting in samples from 2,550 individual animals. These samples were analyzed for presence of BTV antibodies using enzyme-linked immunosorbent assay (ELISA). Each year, 2,550 samples were taken; results showed that BTV antibodies were present in 707 (33.5%) of 2,550 samples taken during 1982, 818 (35.3%) during 1983, and 1,025 (45.8%) in 1984. Serotype identification was not performed in this study, and the authors note that there were no notable trends based on geographical data or cattle age.

Prior studies had discovered BTV seroprevalence in the neighbouring state of Mississippi, with 5.7% in 1977-78 and an increased estimate of 22-34% in the following year of 1979 (Metcalf *et al*., 1980). Additional Louisiana studies examining BTV prevalence found 55% seropositive cattle in 1979 following a local BTV outbreak, followed by 28% prevalence between 1980 and 1981 (Fulton *et al*., 1982).

In addition to the Wyoming incident of 1976, Thorne *et al*. (1988) also described an outbreak of BTV in the same area nearly a decade later. The region of Campbell county had a significant increase in pronghorn deer deaths during August 1984, which prompted examination. One dead pronghorn was sent to the Wyoming State Veterinary Laboratory at the University of Wyoming for necropsy, which revealed evidence of symptoms indicating hemorrhagic disease. Another pronghorn specimen was submitted a few days later, which had similar physical indicators of disease. Pronghorn specimens were analyzed using the same methods as in the 1976 outbreak. It was found that both submitted pronghorn carcasses were positive for BTV-17 via tissue analysis. Details of serum examination are not included in this report. In total, 288 pronghorn, 83 mule, and 13 white-tailed deer were found dead in this region during the 1984 outbreak, and it is estimated that 600-1,000 pronghorn were killed by the virus during the incident.

In British Columbia, no outbreaks of BTV had occurred since its initial discovery in the 1970s. However, a study conducted by Shapiro *et al*. (1991) in 1987 involved a serological survey of 4,610 cattle in the western Canadian provinces to determine if animal sera contained antibodies for BTV and EHDV. Male and female cattle were sampled from areas within the Okanagan Valley, as well as in other southern regions within British Columbia and Alberta, between October 7th and November 27th. Sera were transported to the Animal Disease Research

Institute in Nepean, Ontario where they were screened for presence of BTV and EHDV antibodies using agar gel immunodiffusion testing (AGID). Any sera with initial detection of antibodies were subjected to more specific testing via microtiter serum neutralization (MTSN) and competitive enzyme-linked immunosorbent assay (C-ELISA) to reconfirm. In total, 5 cattle sera tested positive for BTV-11, 125 cattle tested positive for EHDV-2, and 16 cattle tested positive for both types of antibodies. All cattle had originated from the Okanagan Valley except for one with EHDV-2 antibodies. Notably, the cattle's owner had purchased a bull from a farm in Okanagan, and a nearby neighbouring herd had also originated from the area, providing two possible routes by which EHDV-2 could have been transmitted to the seropositive cattle.

Following Shapiro *et al*.'s survey, BTV seropositive animals were once again isolated in the Okanagan Valley, alongside an outbreak of epizootic hemorrhagic disease virus (EHDV) in local deer populations (Dulac *et al*., 1989). A monitoring program was employed by Agriculture Canada in 1988, involving placement of sentinel cattle herds in locations where BTV had previously been contracted by host animals in 1987, as well as in areas that displayed seropositivity during the initial 1970s outbreak. Three of the five sentinel herds displayed seropositivity for BTV and EHDV as of August 19th, 1988, and BTV-11 was confirmed to be present in blood from one of the herds in October. Since this repeated occurrence of the disease, sentinel cattle herds have consistently been maintained in the region to monitor for any further outbreaks of both BTV and EHDV. Sterritt and Dulac (1992) also noted that there were no indications of bluetongue infection outside of Okanagan between 1980 and 1986.

Further examination of these incidents was done by Sellers and Maarouf (1991), who released a short study suggesting that introduction of EHDV-2 and BTV-11 into British Columbia may have been a result of infected *Culicoides* biting midges being carried northward

from Washington state by strong winds. Using wind pattern analysis and weather reports, the authors suggested that high northward winds on August 13th, 1987 may have introduced *Culicoides* carrying EHDV, as this date corresponds with a small outbreak of EHDV-2 in Washington. Northward winds on October 1st may have done the same for BTV infected *Culicoides*, although there were no recorded outbreaks in Washington during this timeframe.

Maps displaying BTV and EHDV occurrences from 1960 to 1969 can be seen in Figures 2.7 and 2.8.

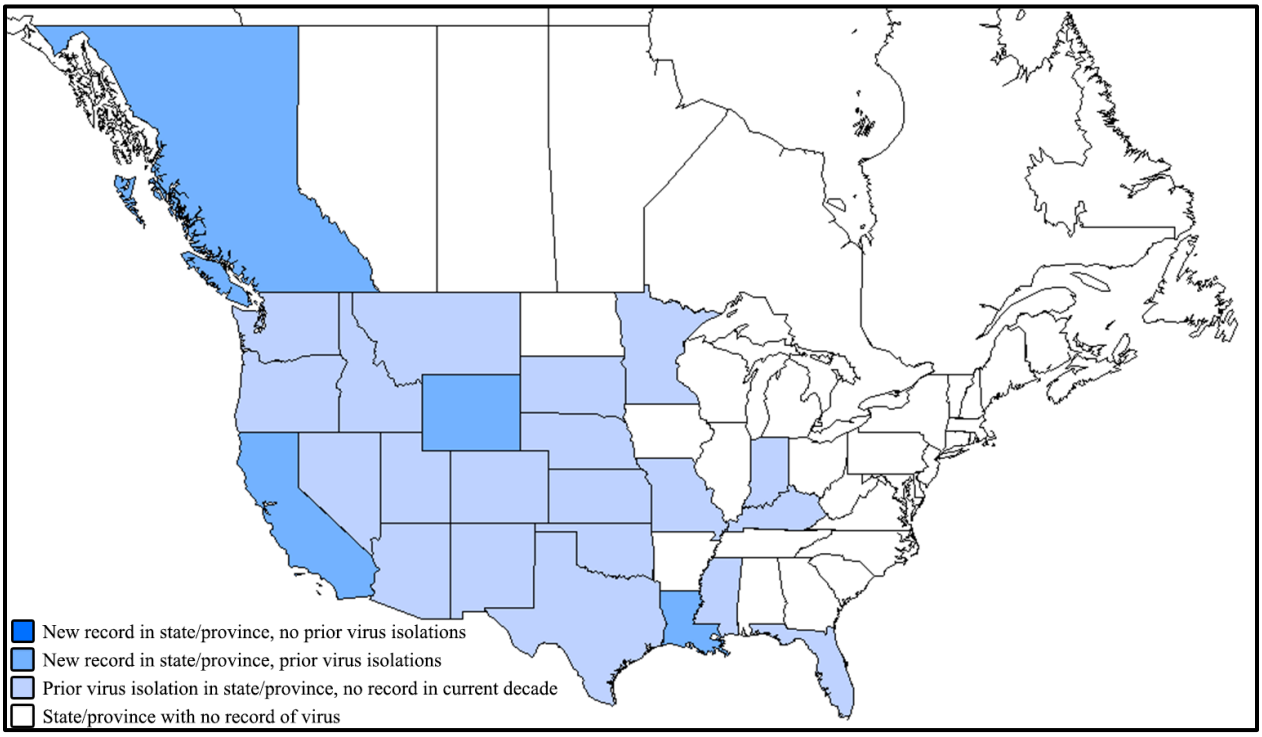

Figure 2.7. State and provincial records of BTV incidents in North America from January 1st, 1980 to December 31st, 1989.

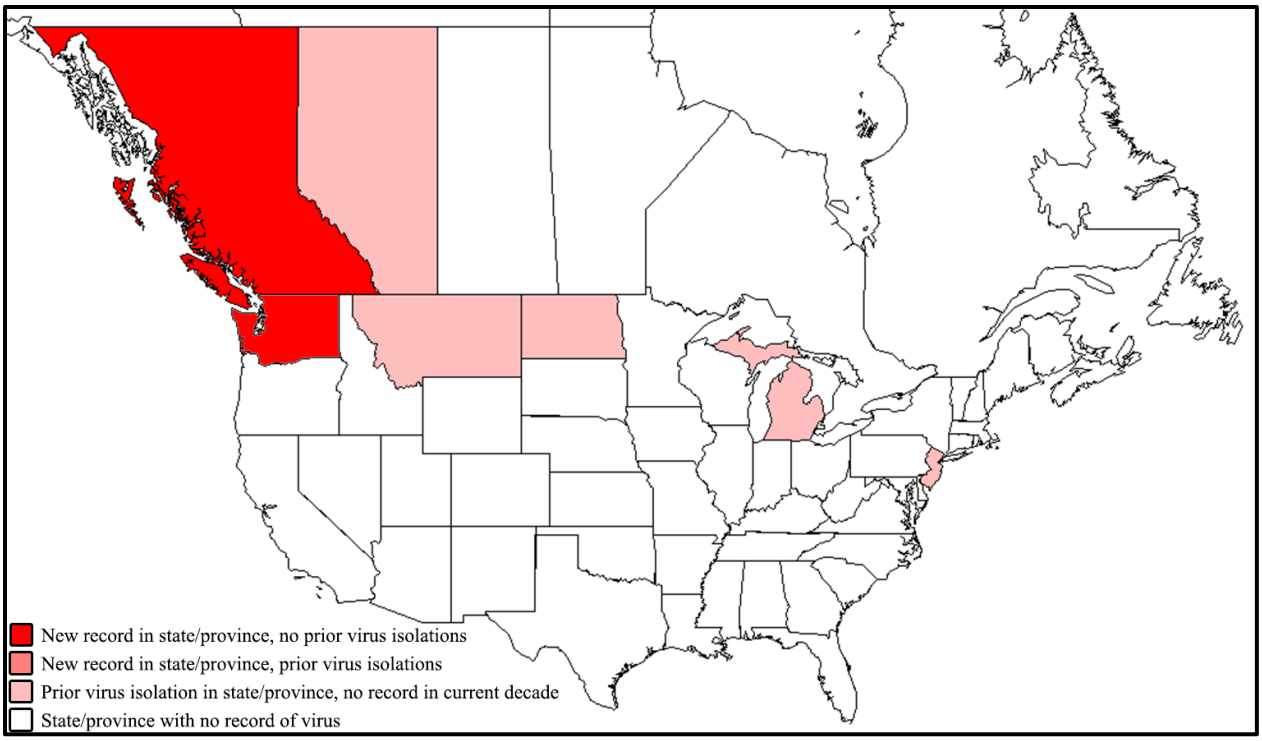

Figure 2.8. State and provincial records of EHDV incidents in North America from January 1st, 1980 to December 31st, 1989.

2.3.2.5 1990-1999: Mass Isolations of BTV and EHDV in Numerous Regions

Between 1978 and 1990, serum samples were collected from 993 California bighorn sheep (various subspecies of *Ovis canadensis* Shaw, 1804) for pathogen testing as reported by Clark *et al*. (1993). Specimens originated from 50 different herds throughout the southeastern mountainous areas of the state, and samples contained blood, serum, mucus, and ear swab material from the sheep. The 50 herds were organized into groups using agglomerative hierarchical clustering procedures, resulting in a total of 14 herd clusters. Testing for 12 different infectious diseases, including BTV and EHDV, was performed at various laboratories in California, Washington, and Iowa. Overall, BTV was isolated from 9 animals, originating from three different clusters containing a total of 430 sheep; EHDV was only isolated from one sheep.

Although overall numbers are small, this study provides further evidence that BTV and EHDV persisted in California throughout the 1980s and into the 1990s.

Ostlund *et al*. (2004) published a report focussing on serological surveys of market-grade cattle throughout the United States between 1991 and 2002. Ten serological surveys for BTV in cattle from 12 different regions were performed within this timeframe. A region was generally defined as a single state, with a few exceptions: these include New England (which combines the states of Connecticut, Maine, Massachusetts, New Hampshire, Rhode Island, and Vermont), Maryland/Delaware, and Pennsylvania/New Jersey. Additionally, Ohio and West Virginia were considered as separate regions between 1991 and 1996, but were combined for later accounts. Other states incorporated into the survey include Indiana, Michigan, Minnesota, North Dakota, New York, Ohio, and Wisconsin. Annual surveys of 600 samples per region were performed in the years of 1991-1996, as well as a less extensive survey in 1997 of only a few regions, and biennial surveys were performed in 1998, 2000, and 2002. Overall, the data showed over 2% bluetongue seroprevalence in Washington, Oregon, Montana, Idaho, Wyoming, South Dakota, Iowa, Illinois, and Virginia throughout all cattle surveys. North Dakota, Indiana, Ohio, West Virginia, New Jersey, Pennsylvania, Delaware, and Maryland all had <2% in some years, but >2% in others; all remaining states involved in the study displayed <2% seroprevalence in surveyed cattle.

In addition to the survey detailed above, Ostlund *et al*. provided an account of BTV-positive samples submitted to the National Veterinary Services Laboratories between 1991 and 2002, as well EHDV-positive isolates. BTV-2, -10, -11, -13, and -17 were all isolated in various states throughout this timeframe, as well as EHDV-1 and -2 (Table 2.2).

Table 2.2. Complete list of BTV and EHDV serotypes isolated in the United States between 1991 and 2002, as listed by Ostlund *et al*. (2004).

State	Isolated BTV Serotypes	Isolated EHDV Serotypes
Alabama	-------	2
Arizona	11, 17	2
California	10, 13, 17	1
Colorado	-------	2
Delaware	Unknown	-------
Florida	2, 10, 13, 17	Unknown
Georgia	10	-------
Idaho	11, 17	2
Illinois	-------	Unknown
Iowa	-------	2
Kansas	11, 17	-------
Louisiana	13, 17	2
Maryland	Unknown	-------
Missouri	13	2
Montana	11	-------
Nebraska	13	2
Nevada	Unknown	-------
New Jersey	-------	1
New Mexico	11, 13, 17	-------
North Dakota	-------	2
Ohio	-------	2
Oklahoma	13, 17	-------
Oregon	11, 17	-------
South Carolina	Unknown	-------
South Dakota	Unknown	2
Texas	11	-------
Washington	10, 11, 17	-------
Wisconsin	-------	2

The first reported and investigated instances of HD in Arizona were described in a 2002 report by Noon *et al*., which details viral analysis of three local deer collected within the state. A dead mule deer was collected in July of 1993 in the Buenos Aires National Wildlife Refuge and was analyzed at the Arizona Veterinary Diagnostic Laboratory (AZVDL). Microscopic tissue analysis showed symptoms consistent with HD, but neither EHDV nor BTV were isolated from tissue samples. A debilitated white-tailed deer was found 2.5 weeks after the first specimen on

August 1, 1993, and was examined in the same fashion. EHDV-2 was discovered using viral analysis methods. Another mule deer was found dead in October 1996 in Hualapai Mountain County Park, after having been observed with severe clinical symptoms the day before. EHDV-2 was also isolated from this specimen.

Further indication of BTV in British Columbia occurred in 1998, when BTV antibodies were detected in 2 cattle from sentinel herds in the Okanagan Valley. While September blood samples were found to be BTV-negative using a C-ELISA test, cattle showed seropositivity for BTV-11 in a subsequent blood sample taken on October 13th. Resampling in November and December confirmed continued seropositivity; all infected cattle were sub-clinical and did not display any symptoms, and the local deer population did not appear to be affected (Clavijo *et al*., 2000). Clinical cases were reported in the Okanagan Valley again in 1999 (AWMDA, date unspecified). These occasional outbreaks of BTV in British Columbia suggest that serotype 11 is well-established in at least the Okanagan Valley, but sentinel herds continue to show success in early detection of possible outbreaks.

Maps displaying BTV and EHDV occurrences from 1990 to 1999 can be seen in Figures 2.9 and 2.10.

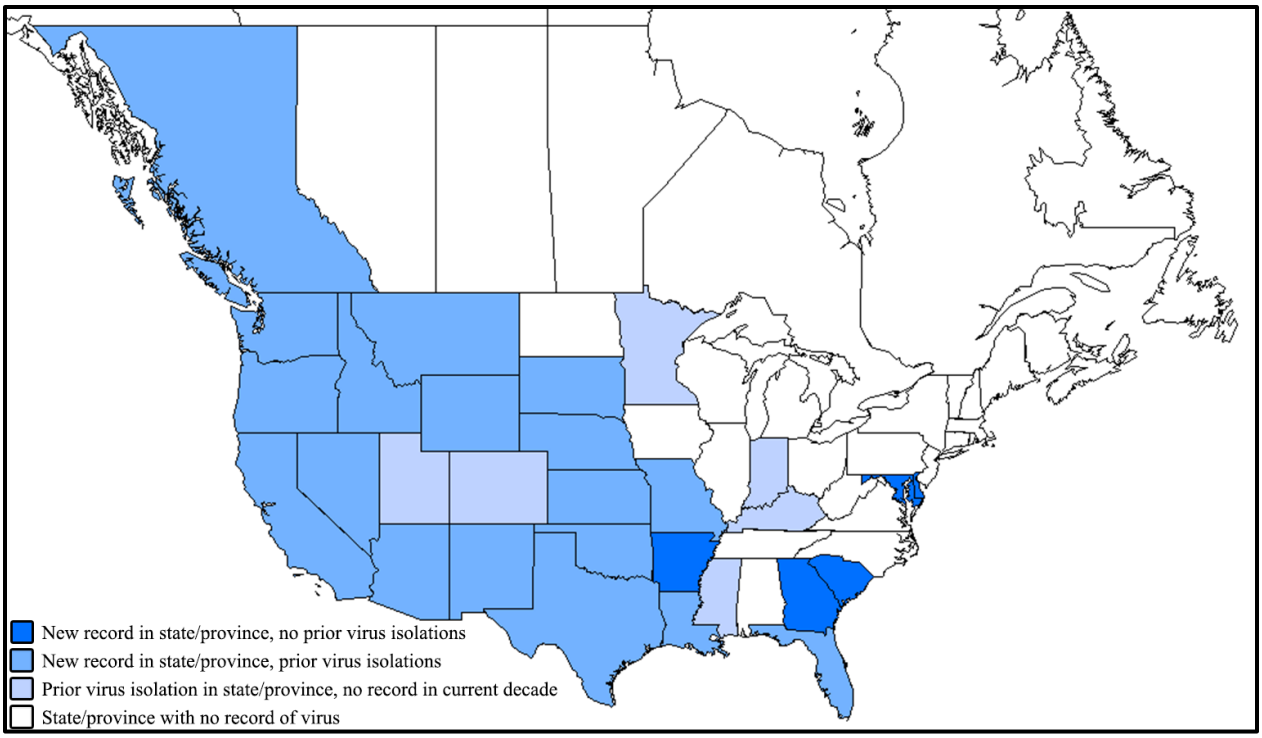

Figure 2.9. State and provincial records of BTV incidents in North America from January 1st, 1990 to December 31st, 1999.

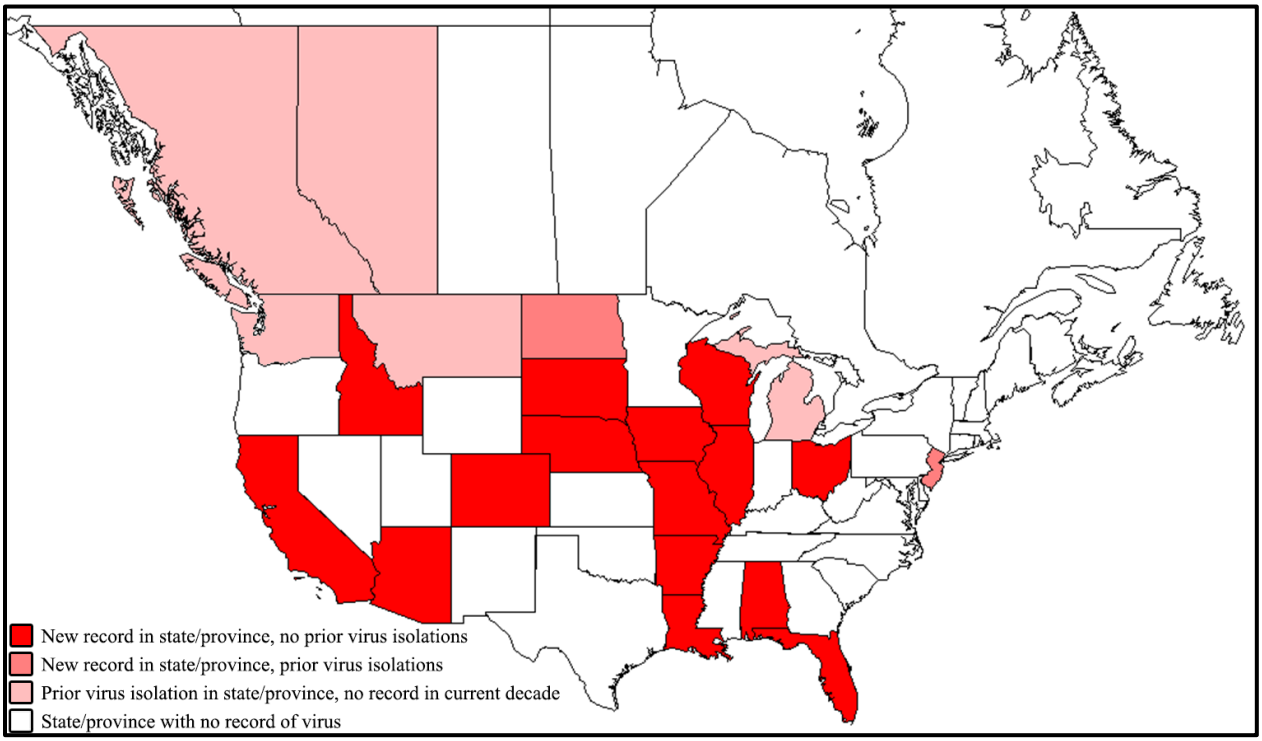

Figure 2.10. State and provincial records of EHDV incidents in North America from January 1st, 1990 to December 31st, 1999.

2.3.2.6 2000-2009: Continued Expansion into New States

An assessment of *Orbivirus* presence throughout Illinois and western Indiana was conducted from 2000 to 2002 by Boyer *et al*. (2010). Sixty cattle herds from 34 different counties were examined for the duration of this study, 52 of which were from Illinois. Serum samples were collected from cattle during the winter months after transmission seasons had occurred, and ELISA tests were used to look for BTV and EHDV antibodies. These results, as well as other variables such as climate and environmental factors, were combined and used to generate risk assessment maps displaying regions with high probability of HD infection. In total, 10,585 cattle were tested for BTV and 9,361 for EHDV; EHDV was found to be significantly more prevalent than BTV throughout the study, with 1,110 (11.6%) and 156 (1.5%) total seropositive animals, respectively. Risk assessment map trends generated by collection and climate data suggested a higher probability of infection by both viruses in more southerly latitudes, with a higher possibility of infection by EHDV in general.

A study performed by Dubay *et al*. (2004) was focused on two infected mule deer, which were once again discovered in Arizona in September 2001, near the town of Prescott. The goal of this research was to determine which serotypes caused infection in the two animals, and to ascertain where initial exposure to the virus occurred. The two mule deer had displayed obvious symptoms of HD, and tissue samples were forwarded to the National Veterinary Services Laboratory in Ames, Iowa for further analysis. Using RT-PCR, one deer was found to be positive for both BTV and EHDV, while the other had only contracted EHDV. Following this discovery, state game management units were notified and asked to gather blood samples from hunted animals. Of the 41 samples submitted, 21 had antibodies for BTV or EHDV; furthermore, of those 21 animals, 4/21 (19%) and 16/21 (76%) displayed seropositivity for EHDV-1 and -2,

respectively. In addition, seropositivity was discovered for all five North American serotypes of BTV, with 12/21 (57%) positive for BTV-2, 15/21 (71%) for BTV-10, 17/21 (81%) for BTV-11, 19/21 (90%) for BTV-13, and 1/21 (5%) for BTV-17. The authors suggest that EHDV-2 and BTV-10, -11, and -13 are endemic to Arizona, as many infected and submitted deer displayed high levels of antibodies to these particular serotypes. They also put forth that presence of certain serotypes would cause local fauna to contract them more frequently, thus resulting in high levels of antibodies in their immune systems.

Another research project examining pronghorn blood samples was conducted by Dubay *et al*. (2006a) to determine factors leading to the decline of species in Arizona. The authors examined fawn recruitment (number of fawns that survive per female deer) in relation to rate of HD infection in does from four different regions within the state. Recruitment records from between 1990 and 2000 were used to estimate fawn recruitment. Aerial observation was used to locate pronghorn and tally males, females, and fawns for each area, at which point the ratio of fawns to females was calculated. The authors noted that recruitment ratios for all four regions, with an average of 20.4 fawns per 100 females, were markedly lower than those of non-epizootic areas within Wyoming documented by Thorne (1988) with an average of 101 fawns per 100 females. Blood samples were collected by hunters from 2001 to 2003 for submission to the Arizona Veterinary Diagnostic Laboratory (AZDVL) located in Tucson, Arizona to analyze presence of diseases by AGID testing, including EHDV and BTV. In total, 139 blood samples were collected and analyzed; EHDV and BTV antibodies were found in 106 samples gathered from all four geographical areas of study. Although it would seem that the low fawn recruitment value may correlate with the relatively high occurrence of HD, the authors suggest that this is not

likely, as actual cases of BTV and EHDV hadn't been diagnosed in any pronghorn deer for a decade.

A small survey for both forms of HD was once again performed in Arizona a year later in 2002, which aimed to evaluate the utility of dried blood samples on paper strips in comparison to traditional serum sample analysis (Dubay *et al*, 2006b). However, a secondary objective of this study was to test serum samples gathered by mule deer hunters for presence of antibodies to BTV and EHDV, and further determine which serotypes were present in the area. Sampling kits were distributed to hunters located in four regions in Arizona, including Yuma, Prescott, Kaibab, and Tuscon. Hunters collected blood samples from hunted deer using the kits and submitted them to designated collection stations for transport to the Arizona Game and Fish Department (AGFD) for study. In total, 42 out of the 125 submitted blood samples tested positive for at least one virus using serum neutralization, and antibodies against all North American serotypes of BTV (BTV -2, -10, -11, -13, and -17) and EHDV (EHDV-1, -2, and -6) were detected, further establishing that all serotypes were present in the state. Paper strips with serum smears were made using 77 out of 125 samples and submitted to the Southeastern Cooperative Wildlife Disease Study College of Veterinary Medicine (SCWDS) for AGID testing to detect BTV or EHDV antibodies; positive sera were further subjected to serum neutralization to discern serotypes. Overall, 35 blood strips displayed presence of HD antibodies, although only 31 of these were in agreement with serum from the same samples. The authors concluded that paper strips are a reliable and simple method to gather blood samples for viral analysis.

In November of 2004, the first instance of BTV serotype 1 in North America was recorded. BTV-1 is suspected to have originated from South Africa; however along with BTV-16, it is also one of the most common serotypes found in China (Zhu *et al*., 2013). A local hunter

in the St. Mary's Parish region of Louisiana killed a white-tailed deer, which had been visibly debilitated before its death. The Southeastern Co-operative Wildlife Disease Study, operating out of Georgia, requested and analyzed deer tissue samples from the region. BTV was isolated from lung and spleen samples, but when the serotype was unable to be determined using tests for known North American BTV serotypes, the tissue samples were further analyzed by the National Veterinary Services Laboratories in Iowa. Virus neutralization testing performed by the NVSL, followed by further RT-PCR analysis, ascertained the presence of BTV-1 (Johnson *et al*., 2006).

In spring 2005, a survey of 549 domestic farm ruminants (460 cattle, 42 goats, 47 sheep) was performed following the discovery of BTV-1. Serum samples were gathered from animals and tested using C-ELISA (complement-enzyme linked immunosorbent assay). Overall, 61 animals were found to possess antibodies to BTV; further testing via virus neutralization assays showed that 20 of these had produced antibodies to BTV-1. Additionally, 6 of these 20 animals displayed significantly higher amounts of BTV-1 antibodies when compared to antibodies of a local serotype, BTV-2 (Barber *et al*., 2005). This report also stated that 9 infections of BTV were detected via PCR in the United States between January 1st and November 2nd, 2005, originating from cattle, deer, and bighorn sheep. Three sheep from Florida were discovered with BTV via viral isolation in October 2005. Mixed infections of both BTV and EHDV were discovered in two deer from South Dakota; BTV-11 was isolated from one of these specimens, but the serotype of EHDV was not determined (Barber *et al*., 2005).

Corn *et al*. (2010) also conducted a study in Arkansas and Kentucky, with data ranging from 1998 to 2006. Their study looked at disease agents that were affecting previously-introduced free-range elk in the two states. Colorado- and Nebraska-native elk had been transported to Arkansas between 1981 and 1985 in an effort to restore the population. Elk were

also collected from six other states (Arizona, Kansas, New Mexico, North Dakota, Oregon, and Utah) and released in Kentucky between 1997 and 2002. Between 1998 and 2006, 170 hunted elk specimens from Arkansas were sampled for blood and tissue analysis; additionally, 44 Kentucky elk were sampled between 2001 and 2004. Samples were tested using ELISA and AGID methodologies. Of all 170 Arkansas samples, 22 elk were seropositive for BTV; 2001 had the highest number and proportion of positive cases, with 12/26 (46.2%) elk testing seropositive. Of the same 170 tested Arkansas elk, 34 displayed seropositivity for EHDV; once again, the highest number and proportion of cases occurred in 2001, with 11/26 (42.3%) elk showing signs of virus. No Kentucky samples displayed seropositivity for BTV, although one EHDV-positive elk was sampled in 2002.

A report published by Allison *et al*. (2010) detailed the first discovery of EHDV-6 in North America, which had previously only been endemic to Australia. From September to October 2006, six different isolations of EHDV were made from white-tailed deer in Illinois and Indiana, USA. The isolates could not be analyzed using typical North American EHDV molecular protocols (for EHDV serotypes 1 and 2), nor did EHDV-1 and -2 primers amplify the viral RNA. Upon this realization, the investigators resorted to antisera and primers that were specific to foreign serotypes of EHDV and subsequently, EHDV-6 was identified from the isolates. Allison *et al*. (2010) also noted that EHDV-6 was discovered in several other states in the following years. EHDV-6 was isolated in Missouri in 2007, and in Kansas and Texas in 2008, suggesting that it was able to persist in the local climate and had become endemic.

An extensive study performed by Roug *et al*. (2012) examined serum samples from black-tailed deer and five different subspecies of mule deer, collected between 1990 and 2007 in California. The purpose of their study was to discern the presence of 6 different diseases of deer,

including BTV and EHDV, and identify geographic regions where diseases are highly prevalent. Serum samples from 943 black-tailed deer and 1,676 mule deer (total = 2,619) were analyzed; samples gathered prior to 1994 were subjected to AGID tests, while C-ELISA was used post-1994 for antibody detection. On average, 13.4% of all samples were found to be seropositive for BTV, and 16.8% were seropositive for EHDV. Unfortunately, these results were not presented on a temporal basis.

A similar outbreak of BTV also occurred in Montana throughout the summer of 2007, in which temperatures were notably higher than usual (Rolston and Johnson, 2012). This prompted a widespread animal quarantine throughout 16 eastern counties in Montana for 30 days. Although this small report provides detail regarding economic costs, unfortunately it does not state which serotype was the cause of the outbreak or highlight any details regarding sampling methods, including specifics about which animals were examined for virus. However, it still suggests that there was a significant BTV event within this timeframe.

It is noted that a widespread outbreak of HD occurred in the state of Kentucky, 2007. A report filed by the Kentucky Department of Fish and Wildlife Resources in 2011 stated that all regions within the state were affected by the outbreak; the report also claims that this outbreak was the most severe incidence of hemorrhagic disease since a BTV outbreak 30 years prior, as discussed in the 1970-1979 subsection. Although occurrences of HD are regularly recorded in the state, 2007 exhibited over 4,000 potential cases in white-tailed deer, although the statistics provided in the report do not specify which cases were caused by BTV or EHDV specifically. Despite this large outbreak, it is noted that the local hunting regulations were altered shortly thereafter to restrict hunters from entering zones with high numbers of deer. This decision resulted in fewer deer hunts and harvests in the following year, and thus the deer population did

not suffer a significant drop, despite the fact that the outbreak had affected so many animals (Brunjes *et al*., 2011).

A minor outbreak of EHDV occurred between August and September 2007 in an ungulate research facility located in Fort Collins, Colorado. Despite displaying no obvious symptoms or prior illness, a white-tailed deer was found deceased in the facility on August 20th. A necropsy was performed on the animal; analysis of the spleen tissue via RT-PCR resulted in positive isolation of EHDV, though no serotype was discerned. An elk was observed with lethargy on August 15th, 2007, but recovered before being euthanized in October for reasons unrelated to sickness. In addition, two more deer were found deceased in September 2007, displaying conditions and symptoms akin to those noted in the first infected deer. Serum samples had been taken from a number of other animals in the facility prior to these recorded deaths, including goats, elk, cattle, and bison. The animals were sampled once more, less than two weeks after the outbreak occurred. Virus neutralization analysis of pre-outbreak serum samples discovered EHDV-1 in one cattle, four bison, and nine goats; EHDV-2 was neutralized from one cattle and three elk. Analysis of post-outbreak serum samples found EHDV-1 in three elk, three goats, and one deer; EHDV-2 was isolated from one deer, two cattle, two elk, three bison, and seven goats. Interestingly, there were no co-infections in which both serotypes were discovered in the same organism (Nol *et al*., 2010).

In addition to the above 2007 occurrences, the United States Department of Agriculture released a report in 2013 focusing on BTV and EHDV presence throughout the country (USDA, 2013). In this report, a number of EHDV outbreaks occurring between 1955 and 2012 are briefly mentioned; however, details are provided on two significant outbreaks in the United States, one of which contributed greatly to animal mortality in 2007. During this EHDV outbreak, numerous

counties within 11 of the midwest and mid-Atlantic states reported over 100, or sometimes over 1,000, dead white-tailed deer in their areas. Prior to this disease incident, the report also notes that the epidemic coincided with high temperatures and drought; this trend was also observed in the second large outbreak in 2012, which is discussed later in this report. EHDV was also isolated in several northern states in the intervening years.

In October 2008, a case of BTV infection was recorded in a female alpaca in a northern California breeding farm. One day prior to examination, the animal had experienced clinical symptoms normally associated with BTV, and died shortly thereafter. The alpaca was then shipped to the School of Veterinary Medicine at the University of California for analysis. Necropsy revealed multiple indications which suggested BTV as a cause, but were not specific indicators of BTV. As such, spleen tissue was extracted and subjected to RT-qPCR to evaluate presence of the virus. Viral RNA amplification and analysis revealed a genetic sequence with 98% similarity to BTV; additional RT-qPCR targeting the NS1 gene confirmed that BTV was indeed present. Serum analysis found that the alpaca had not yet produced any BTV antibodies, suggesting that the animal was killed during the acute phase of the disease. The authors note that this particular case is the first known incidence of camelid death caused by bluetongue in the Americas (Ortega *et al*., 2010).

According to the United States Animal Health Report of 2008, although occasional discoveries and isolations of HD were reported, there were no significant outbreaks of BTV throughout the year. Isolations of BTV throughout the USA in 2008 include BTV-3 from Arkansas, as well as BTV-12 and -17 in Texas. The isolation of BTV-12 constituted the first occurrence of that particular serotype in the United States; prior to this report, BTV-12 had been recognized as a common serotype in various African countries, including South Africa, Egypt,

and Kenya (Rao *et al*., 2015). For EHDV, serotypes 2 and 6 were isolated from samples from Indiana and Kansas respectively, while all three North American EHDV serotypes were isolated from samples in Texas.

The United States Animal Health Association (USAHA) held a committee meeting on November 15th, 2010 detailing BTV and EHDV virus isolations throughout the United States in 2010; however, this document also included details regarding 2009 viral records (Barber *et al*., 2010). Over the course of 2009, 103 virus isolation attempts were performed, resulting in 34 successful isolations. The most prevalent virus was EHDV-2, which was found in 8 states (Alabama, Florida, Kansas, Louisiana, Michigan, Missouri, Ohio, Tennessee), while EHDV-6 was only isolated from a Michigan sample. With respect to bluetongue, BTV-11 was isolated from a Georgia bovine sample, and BTV-17 was found present in Texas. Additionally, the non-endemic BTV-3 was found in Mississippi, and it was also noted that BTV-12 was isolated from a white-tailed deer in Florida, marking the second detection of the serotype since its initial discovery in Texas, 2008, mentioned above (USAHA, 2010).

Maps displaying BTV and EHDV occurrences from 2000 to 2009 can be seen in Figures 2.11 and 2.12.

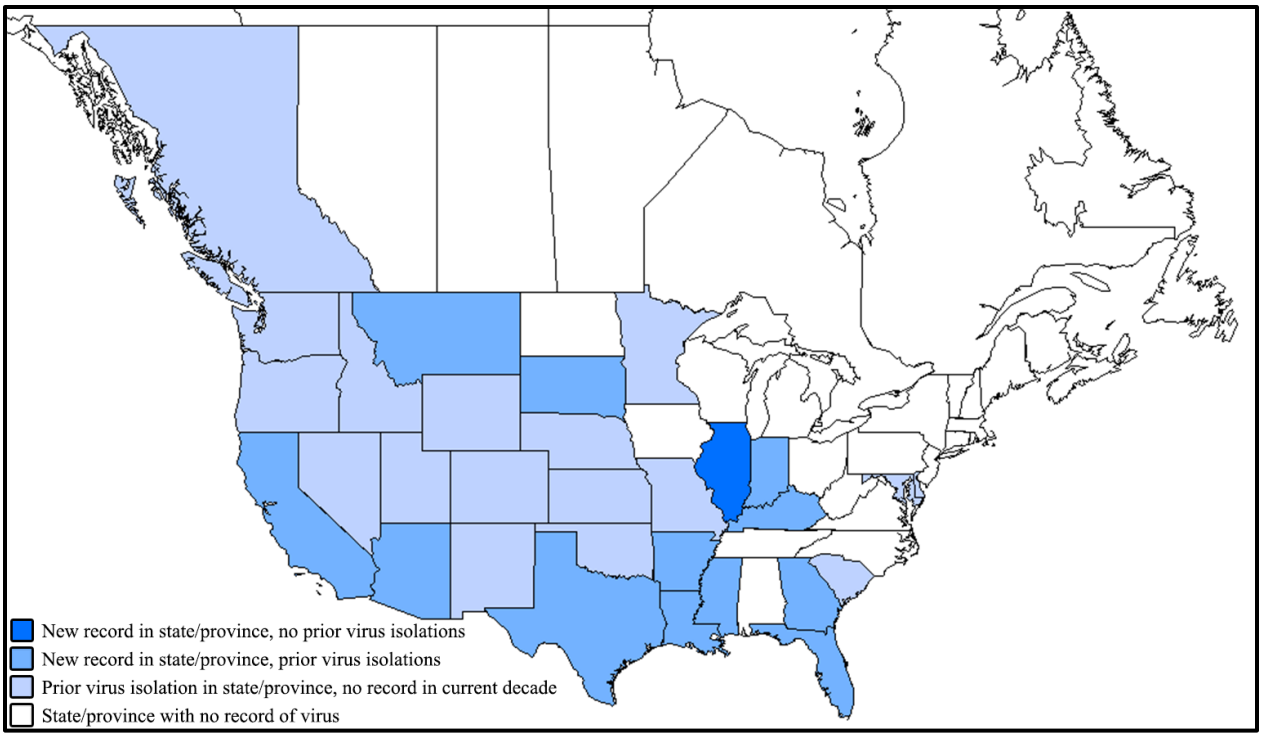

Figure 2.11. State and provincial records of BTV incidents in North America from January 1st, 2000 to December 31st, 2009.

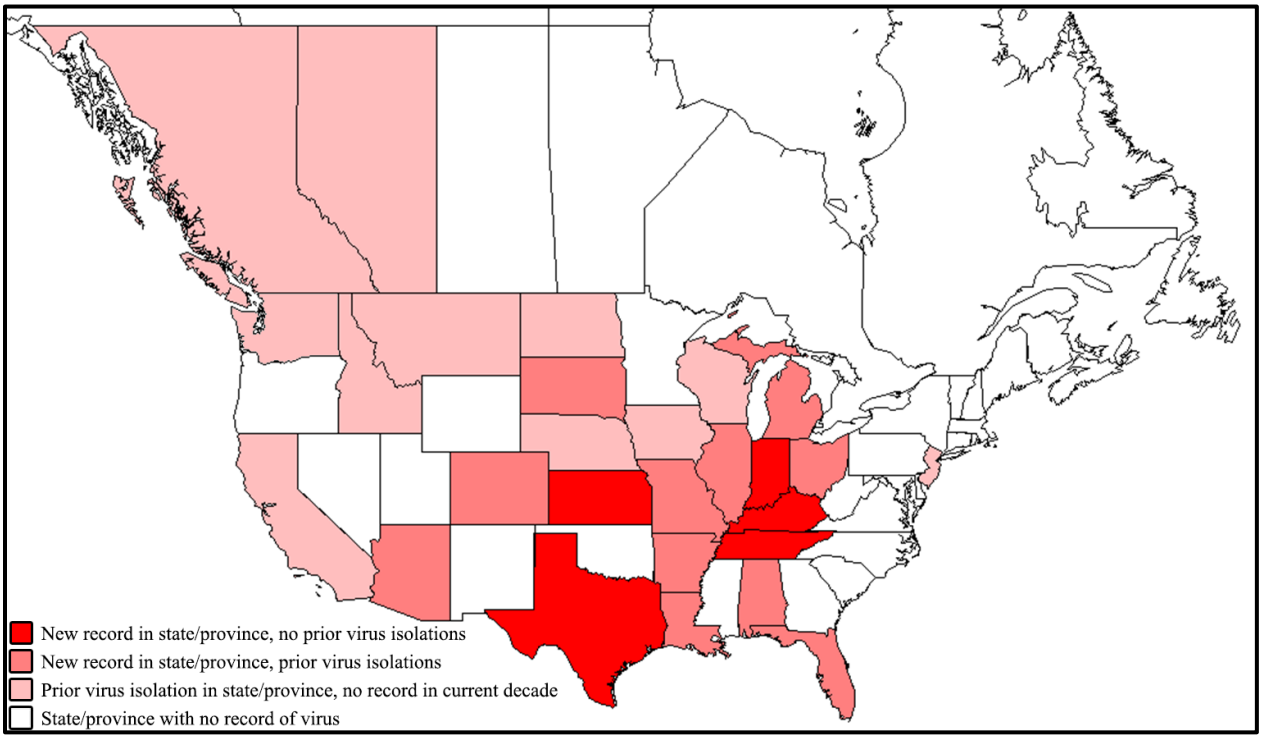

Figure 2.12. State and provincial records of EHDV incidents in North America from January 1st, 2000 to December 31st, 2009.

2.3.2.7 2010-Present: Isolation of BTV and EHDV in Southern Ontario

The 2010 USAHA also detailed BTV and EHDV virus isolations throughout the United States in 2010 (Barber *et al*., 2010). Throughout the year (January 1st to October 31), BTV had been isolated from 11 serum samples. Six cattle in California were infected, four of which displayed seropositivity for BTV-10, while the remaining two were seropositive for BTV-11. In Florida, four sheep were infected with BTV-1, which had not been detected in the USA since its initial discovery in Louisiana, 2004, referenced above. Additionally, BTV-12 (a non-endemic serotype) was isolated from a serum sample taken from a Florida-native deer. EHDV-2 was also found in four deer from Louisiana, Florida, Illinois, and Montana (USAHA, 2010).

Kansas Veterinary Quarterly documents an outbreak of hemorrhagic disease in the eastern regions of Kansas throughout the summer and fall of 2011. Historically, occurrences of hemorrhagic disease had mostly been restricted to the eastern state counties, with very few reports of infection in the western areas. Throughout 2011, a total of 24 counties in eastern Kansas were potentially affected by HD outbreaks. Of these counties, 17 of them had reports of white-tailed deer mortality throughout 2011; the remaining 7 counties exhibited confirmed cases of EHDV-2 from samples of dead white-tailed deer via field necropsies and tissue analysis. The report also mentions that outbreaks had previously occurred in Kansas every 3-6 years on average, with other significant HD events in 1988, 1996, 2002, 2003, and 2007. Reports of dead white-tailed deer as a result of HD have been reported as far back as 1983 in the state (Ruder, 2012).

In 2013, a published thesis focused extensively on the occurrences and distribution of HD throughout Illinois between 2005 and 2011 (Goel, 2013). During this period nearly 5,500 tests

for hemorrhagic disease were conducted in the state; 2,945 of these were BTV tests, while the remainder were for EHDV. In total, there were 262 positive tests for BTV and 85 positive tests for EHDV. Interestingly, a significant spike in positive cases for both BTV and EHDV was observed in 2007, with 86 and 71 seropositive animals, respectively; this may constitute a local outbreak.

In 2011 and 2012, a small number of aborted dog fetuses were examined by the Cornell University Animal Health Diagnostic Center located in Ithaca, New York. In October of 2011, a Rottweiler in Texas aborted a litter of 11 young, one of which was removed via caesarean section. One fetus was sent to the AHDC for examination, where tissue analysis with fluorescent antibody testing (FAT) discovered presence of BTV; further RT-PCR confirmed the presence of BTV-11. In September 2012, two abortion incidents occurred at a bulldog breeding kennel in Kansas, producing 3 dead puppies in total; two of these were submitted to the AHDC for examination. Again, cellular testing and RT-PCR discovered that BTV-11 was present in tissue samples of the aborted dogs. After this finding, the authors attempted to determine the method by which infection of the puppies occurred. The stud dog for the bulldog specimens underwent semen analysis, which was negative for BTV. In addition, the dogs were never fed raw meat that could have potentially been infected with the virus, eliminating the possibility of infection through ingestion. The authors went on to suggest that the dogs contracted BTV-11 through traditional infection by a *Culicoides* biting midge, though BTV infection of canines is thought to be rare (Dubovi *et al*., 2013)

An outbreak of EHDV was observed over a wide geographic range of the United States in 2012 (Stevens *et al*., 2015). Samples of livestock animal tissue and serum were collected by registered veterinarians, from animals that were reported as having visible symptoms by

livestock producers or private farm owners; animals examined in the study included bison, cattle, and captive white-tailed deer. RT-PCR was performed on collected samples using a modified method with only one primer and probe set that is able to detect all EHDV serotypes. In total, 129 cattle and 8 bison were recorded as symptomatic, with most cases described from Iowa, Nebraska, and South Dakota (31, 49, and 37 respectively). Additional seropositive samples originated from Colorado, Illinois, Indiana, Kansas, Maryland, Minnesota, Ohio, Oklahoma, Pennsylvania, Virginia, West Virginia, and Wyoming. Sixty-five white tailed deer were found to be seropositive for EHDV, with most of these cases occurring in Iowa, Michigan, and Ohio (13, 13, and 16 animals respectively).

Briefings presented by the SCWDS further reinforced the suggestions of an HD outbreak in the USA during 2012 (Brewton and Yabsley, 2012). The contents of this report state that the SCWDS received reports of almost 200 occurrences of BTV and EHDV in wild ungulates in 27 states. Serotypes included BTV-10, -11, and -13, as well as all three North American serotypes of EHDV. Notably, the authors observed that although a majority of isolated HD viruses in 2012 were EHDV-2 as in previous years, this was also the first year in which EHDV-6 was found in high abundance. A bulletin released by the National Wildlife Health Center in September 2012 also verified this statement, stating that EHDV-6 presence was observed in Florida, Indiana, Iowa, Michigan, and Missouri, constituting the first major EHDV-6 outbreak since 2006, when it was first detected in the USA (Sleeman and Fischer, 2012).

Mayo *et al*. (2014) conducted research in August 2012 to determine phenological activity of *Culicoides sonorensis* biting midges in California, as well as seasonal patterns of BTV infection in the area. The authors collected insects at a dairy cattle farm in the northern part of the state, using CDC light traps outfitted with sublimating dry ice as an attractant. Traps were set

every other week from dusk until dawn for the entire year, and also throughout the rest of the day during the interseason of December to June, with all trapping concluding in August 2013. Limited trapping with emergence traps was also done between January and March 2014 in two areas where few to no midges were collected during the prior year. Captured *C. sonorensis* were sorted into pools containing a maximum of 20 specimens, homogenized in lysis buffer, and subjected to viral RNA extraction for RT-qPCR analysis. Two pools were found to be positive for BTV. In addition, 35 cattle present (cohort 1) on the farm had been used as sentinel animals beginning in July 2012 to monitor for BTV. The following year, 29 more cattle (cohort 2) were enrolled for the same purpose, and in 2014, 30 more sentinel cattle and 15 calves were enrolled on the farm for BTV detection; all cattle were initially free of BTV infection. Blood samples were collected bi-weekly from each animal, and analyzed using RT-qPCR for viral RNA. In the first cohort of cattle, 33 became infected with BTV between September and November of 2012 (it is noted that two cattle died from complications other than BTV). In the second cohort, only one cattle became infected in June 2013. Of the animals added in 2014, 4 cattle displayed weak BTV infection, and all 15 calves showed seropositivity but displayed negative results for viral analysis using RT-qPCR. It should be noted that the authors did not perform serotype analysis in this study.

In 2013, recent isolations and records of seropositivity for both BTV and EHDV in the United States were discussed at the 116th annual meeting of the US Animal Health Association (USAHA). Throughout 2011, 30 incidents of BTV were confirmed in the country in 8 different states; serotypes responsible for the infections were BTV-11, -13, and -17, and infected animals included sheep, deer, cattle, and one pronghorn. Incidents of EHDV-1, -2, and -6 were also recorded from 8 states, with a total of 23 infections occurring in various deer species, as well as

cattle. Between January 1st and October 15th of 2012, BTV serotypes 9, 11, 13, and 22 were identified from deer, goats, sheep, and dogs, representing 25 cases in total. Additionally, 114 cases of EHDV (serotypes 2 and 6) were reported from Illinois, Iowa, Nebraska, South Dakota, and Virginia from deer, cattle, bison, and elk specimens.

EHDV was found to be responsible for symptomatic infection of cattle in Wisconsin in 2013 (Wisconsin Department of Agriculture, Trade & Consumer Protection, 2013). This short news release distributed on October 3, 2013 did not provide much information regarding the nature of infection, but encouraged farmers to protect their cattle from viral infection by taking preventative action, suggesting that the virus would persist until a hard freeze occurred in the area. The communication also stated that this was the first time that EHDV was found to be present in Wisconsin-native cattle. Cases were originally reported by a private practitioner, and serum samples were sent to the Wisconsin Veterinary Diagnostic Laboratory and Federal laboratory for confirmation of the virus' identity.

A small outbreak of EHDV in Canada was also reported by Pybus *et al*. (2014). From September 5th to 16th, 2013, approximately 50 deer and three antelope were discovered dead in Alberta, in an unnamed region roughly 30-50 km north of the Canada/USA border. The dead animals were generally found near water reservoirs. Of all deceased animals, one deer and one antelope were transported to the Alberta Agriculture and Rural Development laboratory in Lethbridge, Alberta for analysis. Spleen tissues were extracted from both specimens and sent to the Michigan State University Diagnostic Center for Population and Animal Health, located in Lansing, Michigan, for virus detection. Although the antelope tested negative, EHDV was isolated from the white-tailed deer. Further tissue examination at the National Centre for Foreign Animal Disease Laboratory in Winnipeg, Manitoba was able to identify the virus as EHDV-2,

marking the first time this particular serotype was isolated in Alberta in over 50 years. Pybus *et al*. (2014) also mention that EHDV-infected insects may have been brought northward into Alberta from the northernmost United States, describing large northbound wind gusts occurring on August 3rd and September 1st, 2013. This timeframe is somewhat consistent with initial discovery of the dead ruminants in early-mid September.

In August 2015, BTV-13 was discovered in a herd of livestock cattle located within the Chatham-Kent region of Ontario, Canada. During a country-wide survey of cattle herds, the Canadian Food Inspection Agency (CFIA) isolated BTV-13 in the region. The cattle in question had been born and raised locally at the farm where the blood sample was taken, suggesting that it contracted the disease within Ontario. After this finding, province-wide warnings were issued to livestock producers on September 3, 2015 in order to prepare them for the possibility of further BTV incidents. Two years prior to this finding, *Culicoides sonorensis*, the primary North American vector for BTV, was discovered for the first time in Ontario during biting midge sampling at sheep farms throughout the southern regions of the province. The species was recaptured the following year, suggesting that it is able to persist and overwinter in the local climate (Jewiss-Gaines *et al*., 2017). With *C. sonorensis* becoming a permanent resident of Ontario, the possibility of BTV establishment, and a thriving livestock industry in Ontario that is naïve to bluetongue, the potential for a future outbreak should not be discounted.

Later in 2015, the Animal Disease Diagnostic Laboratory of Washington State University published an article confirming a Washington and Idaho BTV outbreak in the fall months of that year (Powell, 2015). Of the animals killed during this event, 42 were transported to the laboratory for necropsy and study; many submitted animals were white-tailed deer, but a number of other ruminant species were also affected, including mule deer, cattle, domestic and bighorn

sheep, and one yak. Tested animals originated from six different counties in Washington, and four counties from Idaho; it is uncertain whether this list contains all affected counties, or only a fraction of them. The report also states that testing for EHDV was negative in all sampled animals, ruling out the possibility of a misdiagnosis.

The first Ontario record of EHDV occurred in September 2017, when two male white-tailed deer were found dead in the city of London (CBC News, 2017). The animals were found on September 12th, at which point they were sent to the Canadian Wildlife Health Cooperative. Symptoms were indicative of EHDV, and serum analysis confirmed the presence of EHDV-2 in the animals. The discovery of the virus was made public on September 27th, and various news outlets issued statements to the public, instructing them to report any findings of dead deer in southern Ontario for subsequent analysis. Interestingly, the timing of this incident coincided with an EHDV outbreak in the Jefferson and Columbiana counties of Ohio, located across Lake Erie from the southern edge of the province (Ohio Department of Agriculture, 2017). It is possible that these incidents were related, as strong northern wind gusts would have a high potential for distributing infected *Culicoides* northward into southern Ontario. In the same document, it is noted that viral incidents have also been recognized in Kentucky and Pennsylvania, both of which share borders with Ohio (Ohio Department of Agriculture, 2017).

Maps displaying BTV and EHDV occurrences from 2010 to present can be seen in Figures 2.13 and 2.14.

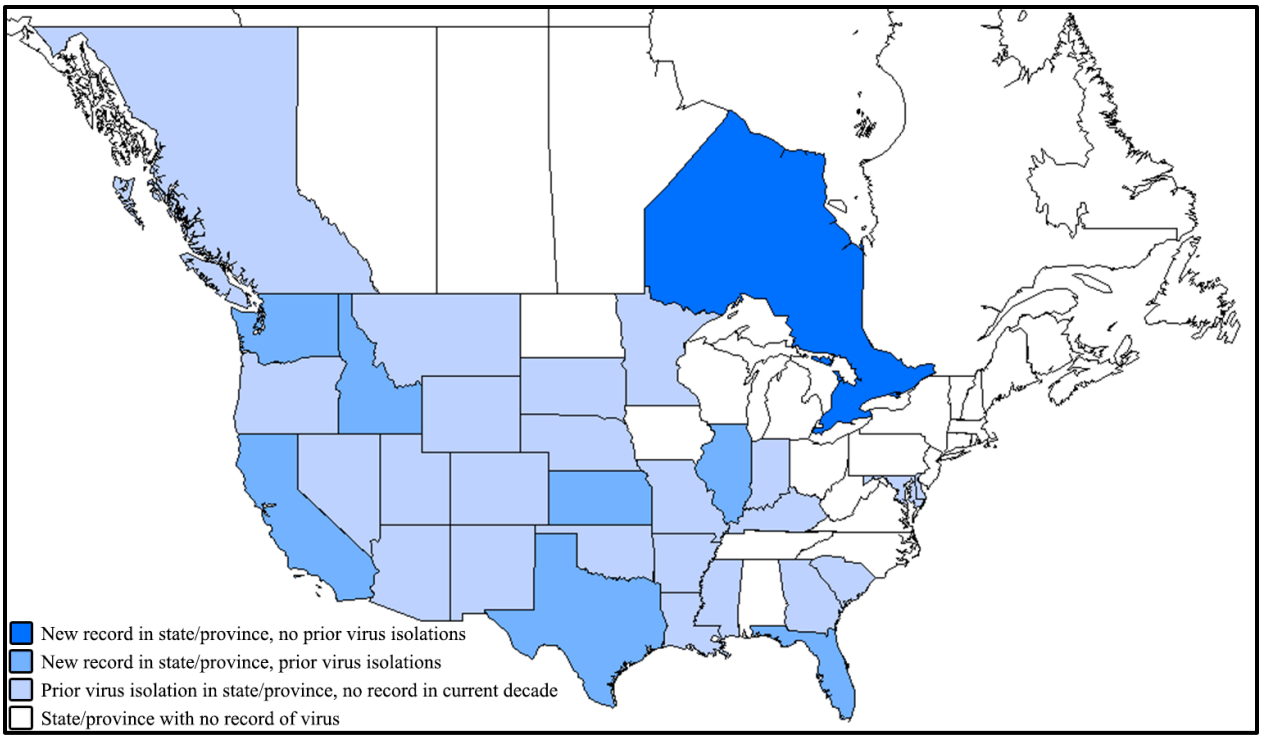

Figure 2.13. State and provincial records of BTV incidents in North America from January 1st, 2010 to date of publication.

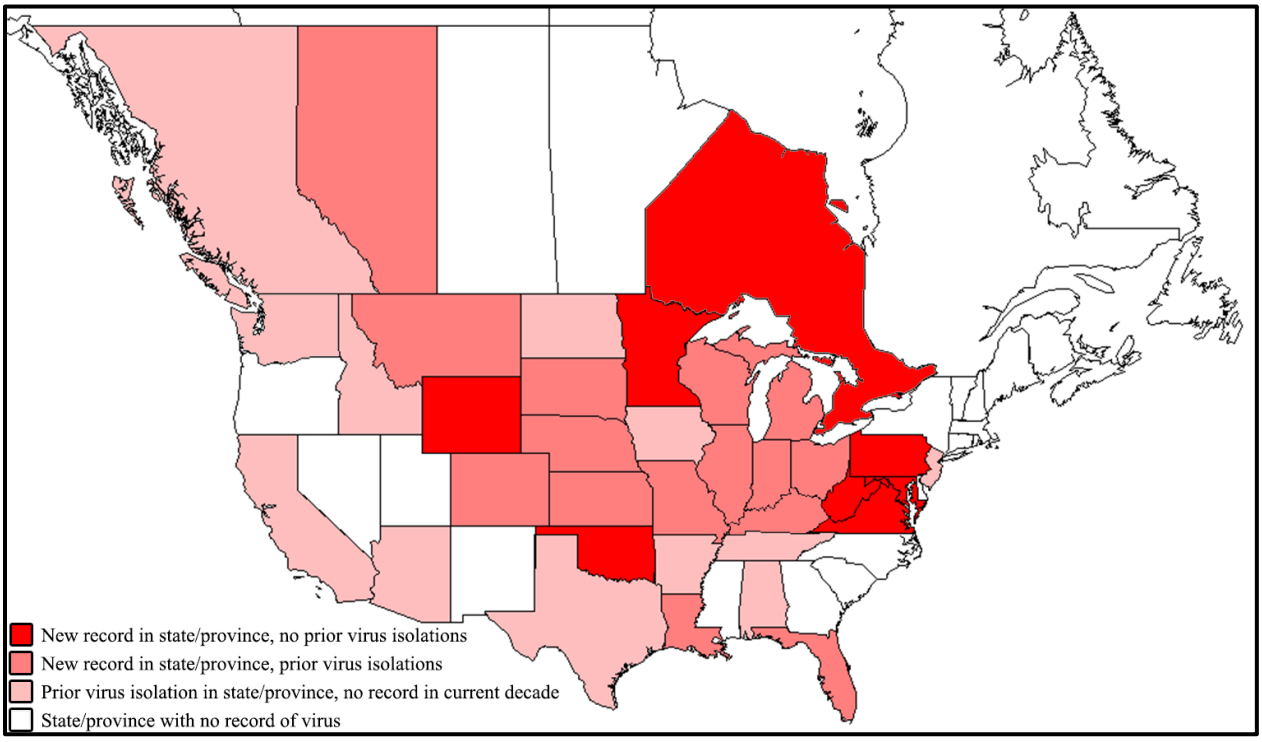

Figure 2.14. State and provincial records of EHDV incidents in North America from January 1st, 2010 to date of publication.

2.4 Discussion

2.4.1 BTV Spread Throughout North America

The overall pattern of new state and provincial records of BTV, from its initial discovery in North America to present day, is displayed by decade in Figure 2.15 (below).

Alongside BTV's initial discovery in California in 1952, cases of illness with similar symptoms and indicators were noted in Texas livestock. These two states may be the disease's true entry point into North America. Furthermore, BTV cases were reported from 11 additional states throughout the course of the 1950s, all of which border one another and exist mainly in the Western half of the continent. Maclachlan *et al*. (2008) noted in their report that the serotype isolated from at least the initial isolate from California was BTV-10. Interestingly, this serotype was also discovered to be the causative agent of the first BTV outbreak in Spain, causing symptomatic disease in over 180,000 sheep and cattle between 1956 and 1960 (Pérez de Diego *et al*., 2014). Though Pérez de Diego *et al*. (2014) suggested that BTV vectors, specifically infected *Culicoides imicola* Kieffer, 1913, may have been introduced from Africa to Spain and Portugal via northerly wind currents, this is not a possibility for North America as it is not geographically proximal to a region where BTV is thought to have originated. As such, it is more than likely that BTV-10 was introduced into the United States via livestock import from a country exhibiting BTV infection. The most likely countries from which infected animals may have been acquired are Africa, which is widely recognized as the origin for numerous initial serotypes of BTV, and Europe. Although BTV outbreaks did not occur in Europe until after the virus was first recognized in North America, it is also possible that the vector and virus were present in Europe prior to the outbreak in Spain and Portugal, but did not cause a significant outbreak until the

latter half of the 1950s. Thus, European livestock farmers and traders may have been naïve to the disease, which is notable for being asymptomatic or very mildly symptomatic in cattle, and shipped infected animals to the US without undergoing proper quarantine protocols. At any rate, it is very probable that BTV was introduced to North America via man-made transportation methods.

Introduction into North America also brought the disease into contact with *Culicoides sonorensis*, a native species within the same genus as the European vector, *C. imicola*. *Culicoides sonorensis* acts as a biological vector of BTV, taking in virus via blood-feeding on infected animal hosts and subsequently producing replicated virus in its salivary glands to pass to another animal it feeds upon later in life. Combined with movement of potentially infected livestock throughout the United States via common methods of transportation, such as the transcontinental railway system established over the course of the previous century, *C. sonorensis* would have strongly facilitated the spread of the virus throughout the 1950s, affecting not only livestock animals, but also wild ungulates such as the various types of deer highlighted throughout this report.

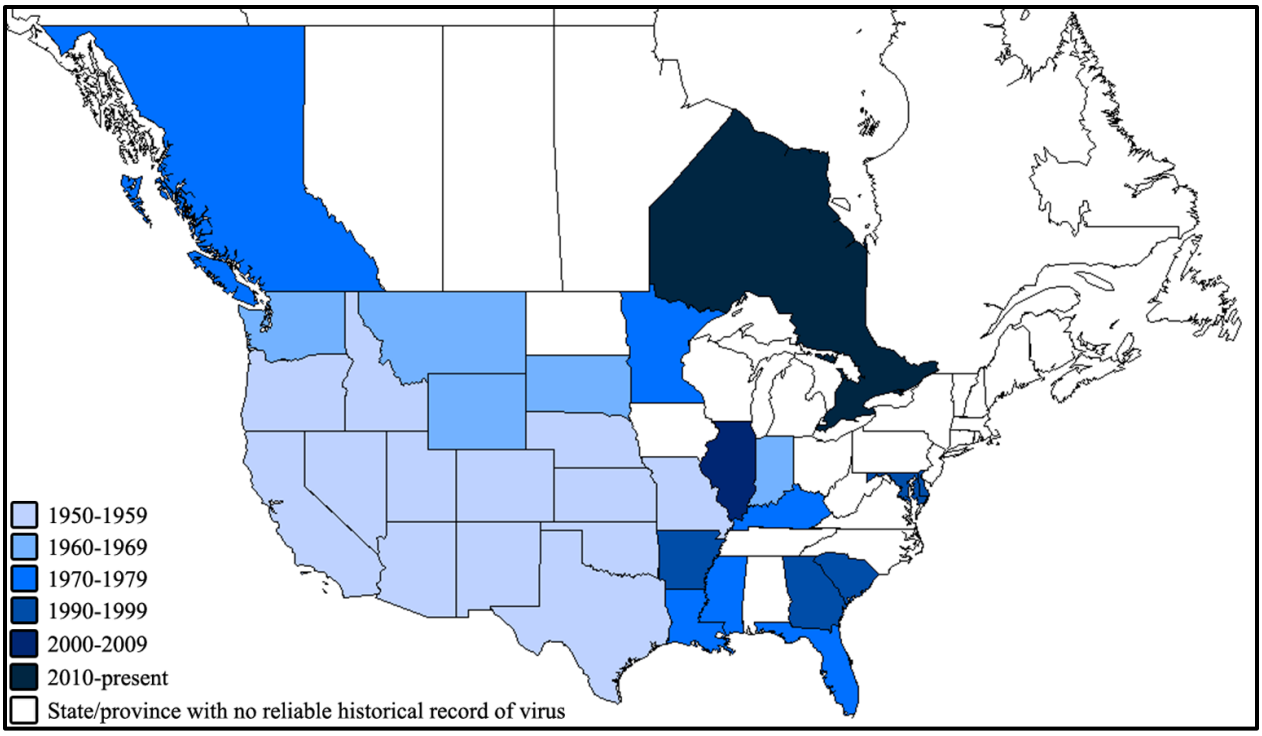

Figure 2.15. Progression of new Bluetongue virus state/province reports in North America, by decade, from 1950 to present. Each time period begins on January 1st of the beginning year of the decade, and ends on December 31st in the last year of the decade. (Note: 1980-1989 not included. Virus was not detected in any new states or provinces during that timeframe)

In the 1960s, BTV continued to reach new regions in the United States, with significant recorded occurrences in Washington State, Montana, Wyoming, South Dakota, and Indiana, the first four of which border the established range of the virus as of 1959. It is unsurprising that those states experienced outbreaks shortly afterward, with livestock transportation occurring between nearby localities experiencing infection (potentially without realizing that infections were occurring), and close geographical proximity which facilitates dispersal of host *C. sonorensis* midges via wind currents or natural expansion of insect populations. The only new state record from an area not adjacent to a state that previously experienced an outbreak was noted from Indiana, located east of Missouri, but with Illinois situated between them. It is interesting that the first recorded outbreak in Illinois occurred approximately 40 years later in the 2000s, despite the fact that the state was bordered by occurrences much earlier. Perhaps farms in

Illinois employed precautionary measures as a result of the outbreaks during the 1950s, preventing their livestock from sustaining a noticeable infection. Alternatively, it is possible that populations of *C. sonorensis* may have not been present in Illinois during this timeframe, and as such the disease would not be vectored within the state.

The 1970s saw BTV emergence in five US states. Three of these states, namely Florida, Louisiana, and Mississippi, are located in the southeastern corner of the country, suggesting that the disease is now endemic to the entire southern region of the country. Though there has never been a significant report, even to the present day, of a BTV outbreak occurring in Alabama, the fact that the disease has been reported in nearly all neighbouring states suggests that it would be able to persist there as long as a vector *Culicoides* is also present. This is a very strong possibility, as *C. sonorensis* is commonly found in Alabama, as well as the states adjacent to it (Borkent and Grogan, Jr., 2009). Additionally, another resident species within the southeast states, known as *Culicoides insignis* Lutz, 1913, displayed vector capability for BTV-2 when Tanya *et al*. (1992) performed vector competence studies on *C. insignis* specimens; this research was a follow up to BTV-2 isolation from *C. insignis* individuals by Greiner *et al*. (1985). Tanya *et al*. (1992) showed that *C. insignis* can develop disseminated infection and transmit the virus to sheep, establishing it as a strong potential local vector within Florida, Georgia, and Alabama, where it has been previously collected (Borkent and Grogan, Jr., 2009). As such, with these two capable vector species endemic to the state, it is likely only a matter of time before a significant BTV outbreak is recorded in Alabama. The remaining two states displaying new records in the 1970s are Missouri and Kentucky, both of which border states that observed significant BTV incidents in the decade prior. With frequent livestock transportation and close proximity between

these states and their adjacent regions, it is unsurprising that the virus was found in these new localities.

One of the most significant BTV occurrences, however, was its discovery in the Okanagan Valley of British Columbia, Canada in the 1970s, only approximately 25 years after its initial isolation in the southern United States, denoting an outbreak at the northernmost latitude of recorded occurrence in North America. With infections occurring throughout multiple years in the mid to late 1970s, this particular incident provided evidence that BTV was able to persist at a high latitude of nearly 50˚N in North America. Another significant BTV outbreak occurred in the province once more in the 1980s. Sellers and Maarouf (1991) postulated that northward wind currents may have been responsible for introduction of biting midges, infected with both BTV-11 and EHDV-2, into British Columbia in 1987-88, facilitating the spread of the diseases and causing resident outbreaks. This possibility is also put forth by Clavijo *et al*. (2000) as a method by which the initial outbreak in the 1970s occurred. Prior to this discovery, BTV outbreaks had been recorded from Washington State, Idaho, and Montana, all of which are US states that border British Columbia's southern edge. Therefore, it's feasible that any sort of strong northerly wind pattern may have brought infected ceratopogonids along with it, introducing them to naïve animals and causing infection.

BTV was not recorded from any new states or provinces in the 1980s, though it was observed affecting livestock in British Columbia, Louisiana, Wyoming, and California in the span of the decade. It wasn't until the 1990s that the virus emerged in new regions of the United States, with significant infections recorded from Arkansas, Delaware, Georgia, Maryland, and South Carolina, alongside numerous repeat infections in states with prior incidents. Infections occurring in Arkansas, Georgia, and South Carolina at this timeframe are unsurprising; Georgia

and South Carolina are near or adjacent to the state of Florida, and with the occurrence of strong sub-tropical winds, human transportation between neighbouring states, and the presence of two BTV vectors as mentioned above, these states were at a high risk of infection the moment Florida showed evidence of BTV presence. Delaware and Maryland are more surprising cases, however, as they lie on the eastern coast of the USA, with Kentucky as the closest state with prior BTV incidents. It is feasible that human transportation may have brought infected insects or cattle from a nearby state into Delaware and Maryland, with wind patterns being a less likely method of entrance. Theoretically, if wind were displacing infected vector flies outward from a state such as Kentucky, it is likely that BTV infection would have been noted in the states adjacent to it beforehand, such as Virginia and West Virginia, especially over the span of the 1980s in which no new state records were even observed. As such, infection may have been introduced into Delaware and Maryland via human activity, with livestock transportation being a probable method.

In the 2000s, the only new incursion into a previous uninfected state was noted in Illinois. Though it is unsurprising that Illinois would eventually harbor infected animals, since it is bordered by three states (Indiana, Kentucky, and Missouri) that exhibited BTV infections in previous years, what comes as a surprise is that it took so long to experience a significant incident. It could be that Illinois farms were proactive with respect to reducing chance of infection, or perhaps they experienced asymptomatic infections that went unnoticed.

In the 2010s, Ontario observed its first BTV incident, currently constituting the only significant range expansion within the decade. The isolation of BTV in Chatham-Kent county in 2015, as well as the discovery of *C. sonorensis* within the province only 2 years prior, suggest that there is significant potential for the virus to reach additional areas of Ontario. This would

have significant implications, as the southerly latitudes of the province contain numerous farms with cattle, sheep, and goats as livestock, all of which are likely naïve to the virus unless they were transported into the province from an area that previously experienced a BTV outbreak. Additionally, if both the vector and disease manage to spread more northward, Ontario could sustain significant BTV outbreaks in white-tailed deer populations. Left unmonitored, and with less of a focus on vaccination of deer as opposed to livestock animals, this could have serious impacts on provincial deer numbers.

Overall progression of BTV throughout the continent shows a north-eastern movement, with initial discovery in the south-western region of the USA, and the more recent incidents in Canada and near the eastern coast of the United States. BTV incidents have occurred in the latitude range that encompasses the United States, and as such it could become endemic to every state in the country if the host and vector organisms are present within those states to facilitate disease replication and spread. Additionally, with the existence of BTV and *Culicoides sonorensis* at latitudes of at least 50˚N (as with the BTV outbreak in Okanagan Valley of British Columbia), it is possible that the virus could persist and cause outbreaks throughout every Canadian province. For the time being, this would likely exclude the state of Alaska, as well as the Canadian territories (Yukon, the Northwest Territories, and Nunavut) as they are at more northerly latitudes where *C. sonorensis* is not likely to occur. Hawaii is also an unlikely state that would experience an outbreak, unless infected organisms (livestock or insects) are taken there via human transportation.

2.4.2 EHDV Spread Throughout North America

The apparent pattern of EHDV movement in Canada and the United States over the course of the last 60 years is not as clear-cut as the observed pattern of BTV, as seen in Figure 2.16 below.

The first recorded incidents of EHDV were noted in the 1950s, the same decade in which BTV was first discovered in the United States. However, initial findings of EHDV occurred in North Dakota, Michigan, and New Jersey, three states which are located in the north to northeast regions of the country, as opposed to the southwesterly origin points seen in the case of BTV. Interestingly, EHDV is thought to have originated in North America, as it is the first country in which the disease was isolated and described, unlike BTV which originated in Africa. Thus, it is probable that EHDV was present on the continent prior to these events, at least to some degree, and affecting animals before being recognized and isolated. *Culicoides sonorensis* has been noted as the most efficient transmission vector for EHDV; however, the species was not thought to occur in any of these three states as it was never captured there, up to as recently as 2009 (Borkent and Grogan, Jr., 2009). Despite this observance, EHDV has thrived in the northern states for decades, using some form of insect vector to propagate. There are two likely scenarios that may explain this inconsistency. Firstly, it is possible that EHDV is vectored by an alternate organism (likely one or multiple species of *Culicoides*) in the northernmost states, allowing it to infect deer and livestock. The other possibility is that *Culicoides sonorensis* has always occurred in these regions, but may have been misidentified upon capture as its sister species *Culicoides variipennis* Coquillett, 1901. These two species are superficially very similar, and without modern methods of identification such as DNA barcoding, it is possible that entomologists dismissed *C. sonorensis* specimens as *C. variipennis*. It should also be noted that Borkent and

Grogan Jr. (2009) list *C. variipennis* as a species widespread throughout North America, ranging from British Columbia to Nova Scotia and southward. Perhaps, amidst the surveys for Ceratopogonidae in the country (of which there would likely only be very few, as this family is not studied by many entomologists or taxonomists), some of these widespread *C. variipennis* were instead *C. sonorensis*. This also reverberates with the first possibility of an alternate vector, as if *C. variipennis* was in fact the only species present in those particular states at the time, perhaps it is capable of vectoring EHDV to a significant degree.

In the 1960s, the only significant outbreak was the first Canadian isolation of EHDV-2 recorded in the province of Alberta. This particular serotype has since been considered endemic to the province, and with the frequent observance of *C. sonorensis* in this region, it is unsurprising that EHDV is capable of occurring there. Pybus *et al*. (2014) have suggested that northerly winds from the states may carry infected midges along with them during outbreaks in the northernmost United States, facilitating infection within Alberta. This is somewhat verified by a recorded EHDV outbreak nearly 50 years later, but the only northerly outbreak occurring within geographical and temporal proximity to the Alberta outbreak of 1962 was an incident in North Dakota in the decade prior, a state which does not lie directly adjacent to the province in question. This does not necessarily exclude wind as a factor, but it does limit the possibility that it was an important component in this incident.

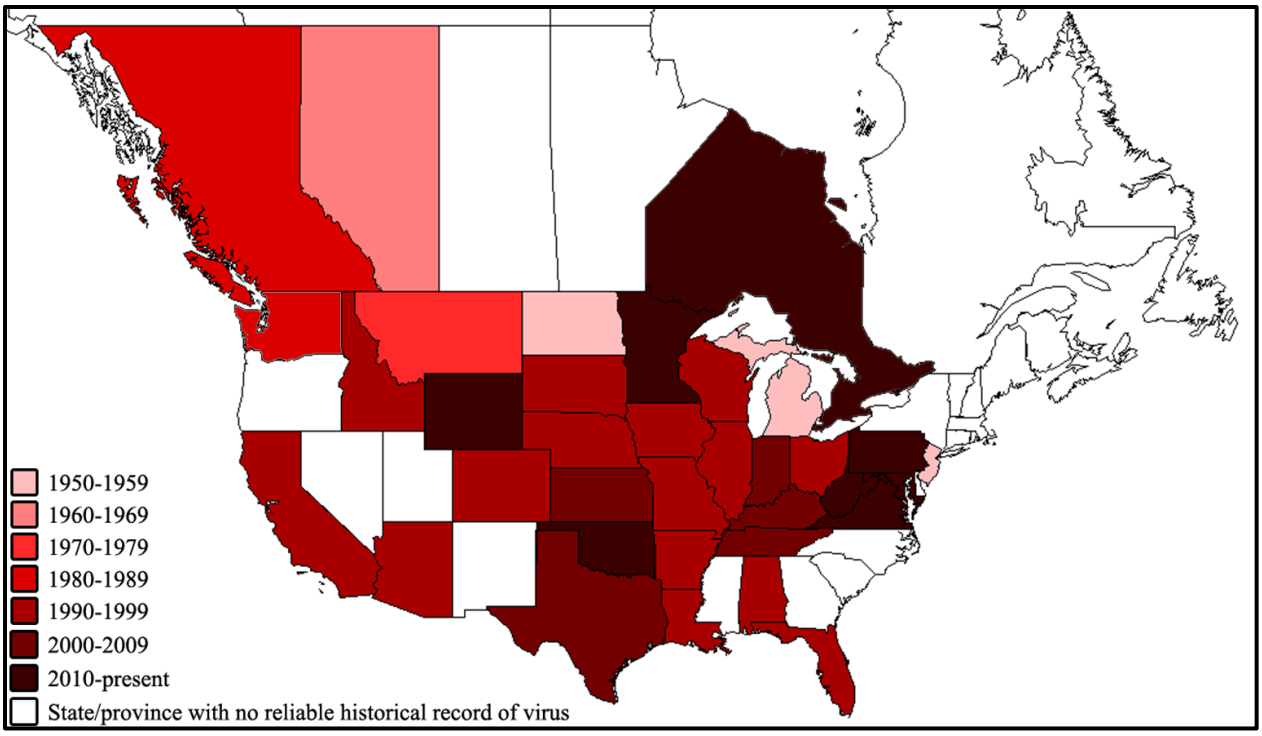

Figure 2.16. Progression of new Epizootic Hemorrhagic Disease virus state/province reports in North America, by decade, from 1950 to present. Each time period begins on January 1st of the beginning year of the decade, and ends on December 31st in the last year of the decade.

Between 1970 and 1979, there was again only one significant outbreak of EHDV constituting an apparent range expansion, this time occurring in the state of Montana in the latter half of the decade. With two regions bordering it that had previously experienced outbreaks, it was somewhat predictable that Montana would experience EHDV infection shortly thereafter. However, this pattern of occurrences seems to go against the postulation put forth by Pybus *et al*. (2014), implicating northward winds as a method of introducing infected flies into Alberta, as no outbreak is recorded from the province again until the 2010s. With such a sizeable outbreak in Montana, by this logic an outbreak should have occurred in Alberta shortly afterward.

The 1980s saw EHDV outbreaks in two new regions: British Columbia, Canada and Washington State, USA. Sellers and Maarouf (1991) put forth the possibility that, during the small outbreak in Washington during October August 1987, infected midges were carried

northward by wind patterns, facilitating the introduction of EHDV into British Columbia, which occurred roughly two months later in October and persisted until November. With the timing of these outbreaks, this certainly appears to be a possible method by which EHDV was introduced into BC. However, it should also be noted that EHDV infections were occurring in Montana throughout the decade prior, and Alberta the decade before that, both of which border the province. As such, it is also feasible that infected insects made their way into BC from these other origin points, or that infected livestock were transported into the province, facilitating spread of virus by local *C. sonorensis* populations. The method by which EHDV was introduced to Washington state may also be attributed to wind currents sweeping *C. sonorensis* from Montana, which, though it does not directly border Washington, lies extremely close to Washington at its western-most edge. Proximity between these states also implies simple ground-based transportation between them, and so livestock transport into Washington may be a possibility as well.

Throughout the 1990s, many reports and studies were produced of EHDV occurring in states where it was previously unrecorded, ranging from the northernmost to southernmost latitudes and from the eastern to the western coast. New EHDV records in the US between 1990 and 1999 include a total of 15 states: Alabama, Arizona, Arkansas, California, Colorado, Florida, Idaho, Illinois, Iowa, Louisiana, Missouri, Nebraska, Ohio, South Dakota, and Wisconsin. It is surprising that the disease would seemingly appear in such a widespread pattern so suddenly, but there are at least a few possibilities that may explain this trend. The first possibility is that EHDV was actually endemic to all of these states beforehand, but did not cause a significant and recognized infection until the 1990s. Perhaps only asymptomatic infection in livestock and sporadic deer deaths occurred prior to this, which may not have been perceived as a disease

outbreak. Other possibilities may involve range expansions of the vector, *C. sonorensis*, or a dramatic increase in transportation of livestock throughout the country without proper quarantine measures, resulting in infected animals beginning the viral infection cycle in new regions. These explanations do not appear as strong, as *C. sonorensis* was likely already widespread throughout the United States by this point, and there is also little chance of animal transport increasing so drastically between the 80s and the 90s, particularly in such an irresponsible manner that would cause mass introduction of EHDV to so many new states. For an unrecognized disease, this may be a possibility; however, with EHDV as a recognized viral agent since 1955, this type of spread by human methods does not seem probable. Therefore, the most likely explanation appears to be that EHDV occurred naturally near or within states displaying new records, but went unrecognized until this decade.

In the 2000s, EHDV was isolated in five more US states, including Indiana, Kansas, Kentucky, Tennessee, and Texas. It should also be noted that all states with new EHDV records lie adjacent to one or multiple states that showed prior infection to virus, and which also exhibited re-infection of EHDV during the 2000s. The observation of outbreaks in such close geographical and temporal proximity to newly-infected states suggests that states exhibiting new virus records likely had infected insect populations expand within their boundaries, or may have been distributed via wind patterns or human transportation. The 2010s saw an additional 7 states displaying indications of EHDV introduction, with new state records in Maryland, Minnesota, Oklahoma, Pennsylvania, Virginia, West Virginia, and Wyoming. Once again, all 7 of these states bordered areas showing prior infection or which exhibited a reoccurrence of EHDV during the same decade, and so it is unsurprising that infections were eventually observed within these new regions. As such, it is likely that EHDV was introduced via similar methods as discussed for

new state records in the 2000s. Ontario also exhibited its first EHDV infection in white-tailed deer in 2017. This finding, though important, is not terribly surprising, considering the discoveries of *C. sonorensis* in 2013 as well as BTV in 2015 within the province, suggesting that a virus so similar to BTV would also be able to persist. It should be noted that this occurrence coincides with EHDV outbreaks in the neighbouring states of Michigan and Missouri, as well as outbreaks in Ohio, which is located just south of Ontario, across Lake Erie. It is possible that infected vector insects entered Ontario from Ohio via wind currents bringing them across the lake, which would place them near London; another possibility is that they were transported into the province via one of the many road borders nearby, located at Windsor and Sarnia.

The seemingly less organized emergence of EHDV (as compared to the general north-eastern progression as observed in BTV) throughout North America is strongly indicative of the possibility that the virus was widely endemic in many different regions, but was unrecognized until much later. The vast geographical ranges at which EHDV was observed already encompass the latitude range of the United States, and with the disease so widespread at present, it is probable that the virus can exist in a very similar range as BTV: every US state excluding Alaska and Hawaii, as well as the Canadian provinces excluding Yukon, Northwest Territories, and Nunavut. Of course, this is dependent upon a competent vector being present, and as such must co-ordinate with *C. sonorensis* populations, or the geographical range of another yet undiscovered viral vector in the northernmost regions.

2.4.3 Additional Notes Regarding Virus Detection and Distribution

In addition to the aforementioned methods by which viral dispersal may have occurred, there are other possibilities that should be considered.

Interestingly, there was a spike in EHDV reportings in the 1990s, although EHDV was first detected in the 1950s. New records in 15 states were reported in the 1990s. It appears unlikely that the virus reached all of these states within the same timespan, and instead it may be that EHDV existed unnoticed in wild ruminants. The sudden surge of EHDV detection may be the result of enhanced knowledge dissemination in the 1990s rather than true disease spread via natural or anthropogenic causes. In contrast, reports of BTV were noted in many states, even in the first decade of its discovery, probably because of its devastating effects on livestock such as sheep (which would not likely go unnoticed). In this respect, it appears that knowledge transfer of BTV was rapid and effective.

The known distribution of the vector, *Culicoides sonorensis*, has possibly been limited as a result of poor recognition of the species, as well as confusion of *C. sonorensis* specimens with one or both of its sister taxa (*C. variipennis* and *Culicoides occidentalis* Wirth and Jones, 1957). Throughout prior discussion of results, trends are discussed that may result in introduction of *C. sonorensis* specimens into new areas, subsequently facilitating BTV and EHDV spread through midge bites. However, it is possible that the species merely went unrecorded or misidentified throughout North America, and had a wider species distribution throughout the continent than originally thought. The maps presented throughout this report, as well as the theory presented by multiple authors that *C. sonorensis* is the primary North American vector of both BTV and EHDV, may suggest that *C. sonorensis* was widespread throughout the continent for the last 60 years, with the presence of the disease in local animal populations being the true limiting factor.

Thus, simple introduction of an infected animal, such as a cattle or deer, could result in dispersal of BTV or EHDV through local *C. sonorensis* specimens that had gone unrecognized. Though this is an uncertainty, and would require extensive retrospective research of *Culicoides* identification throughout the latter half of the 20th century at the very least, the possibility of such misidentifications should be reduced via taxonomic or molecular identification whenever possible, particularly for species capable of harbouring animal and human viruses.

2.5 Conclusion

An extensive literature search was performed for scientific articles, news items, and reports containing details regarding significant outbreaks or occurrences of bluetongue virus (BTV) and epizootic hemorrhagic disease virus (EHDV) in the United States and Canada since their discovery in North America in the 1950s. It was found that, by the time of submission of this report, BTV had occurred in 29 US states and 2 Canadian provinces, and exhibited an overall northeastern progression from Texas and California, in which it was originally discovered, to numerous states on the eastern coast and as far northward as Okanagan Valley in British Columbia. This seemingly clear trend suggests that BTV radiated outward from its emergence points via transportation methods carrying infected animals or *C. sonorensis*, or by weather patterns distributing infected *C. sonorensis* via wind gusts and currents, to which they are highly susceptible. The emergence of EHDV throughout North America displayed a less distinct pattern of emergence, with initial occurrences in 3 northeastern states in the 1950s, but further outbreaks appearing in a more scattered trend. Spread of EHDV from one state or province to an adjacent region would likely have been caused by weather patterns or human

transportation; however, the mass occurrence of EHDV infections during the 1990s in many of the US states suggests that the virus may have been endemic to those states, but had previously gone unrecognized as a significant ungulate disease. Extrapolating from the current geographical ranges and history of BTV and EHDV occurrences, it seems likely that both viruses could become endemic to nearly every Canadian province and U.S. state given enough time, a competent insect vector, and a climate in which the vectors and viruses could persist and flourish.

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Chapter 3: A Survey of Southern Ontario Sheep Farms for Biting Midges (Diptera: Ceratopogonidae) and Bluetongue Virus

3.1 Introduction

Canada is well-recognized for having a large farming industry, including the maintenance of livestock for meat and dairy products. Livestock animals, such as sheep, goats, and cattle, are frequent blood-feeding targets for biting insects. This is partly due to the fact that these animals can frequently been found in large flocks or herds, and as such they produce a large amount of carbon dioxide that acts as a chemical attractant for many biting insects (Venter *et al*., 2016). Additionally, large herds of livestock can be extremely susceptible to vector-borne diseases, particularly if the herd or flock has not had prior exposure to a specific pathogen. If animals are exposed to a new virus, parasite, or other pathogenic microorganism, it is more likely that they will display symptoms, as they would not have developed antibodies to properly respond to the threat. There is a high probability of disease spread throughout an entire herd of animals that are constantly housed together, as if one member of the herd is infected with a disease, insect vectors could potentially transfer that disease to other nearby hosts.

One such pathogen, bluetongue virus (BTV), has yet to be considered officially established in Canada. There have been occasional instances of BTV reports within our country, the most notable of which occurred in the Okanagan Valley of British Columbia (Thomas *et al*., 1982). However, there is no evidence that these outbreaks led to permanent establishment. That being said, with 5 different serotypes present in North America, BTV is quite prevalent across the continent (Walton, 2004). Additionally, with the gradual environmental shift coming about due to climate change, new diseases may become able to thrive and establish outside of their known ranges of distribution. Thus, since BTV has been able to exist at latitudes near-equivalent to those of Canada in Europe, and even in British Columbia for a brief period, it is not outside the realm of possibility that it could thrive in the southern range of Ontario.

In order for BTV to establish itself, however, a vector organism must also be present that is capable of transmitting the virus. Members of the genus *Culicoides* (Latreille, 1809; Diptera: Ceratopogonidae) have been known to vector a number of livestock viruses, including vesicular stomatitus virus (VSV; Drolet *et al*., 2005), epizootic hemorrhagic disease virus (EHDV; Maclachlan and Guthrie, 2010), and BTV. In North America, the primary BTV vector is *Culicoides sonorensis* Wirth & Jones 1957 (Carpenter *et al*., 2015). Previous records of this species have been made in a number of the United States, as well as the western-most Canadian provinces of British Columbia and Alberta (See Chapter 2); as of this study, *C. sonorensis* had not yet been discovered in Ontario. However, the possibility of vector jumping is not unrealistic, as this has been observed before in previous BTV outbreaks within Europe, where local species began to spread the virus after it was introduced from African biting midges (Pérez de Diego *et al*., 2013).

The purpose of this study was to trap biting midges at sheep farms throughout the southern regions of Ontario and assess the presence of vertebrate-feeding genera that may pose a threat to the farming economy. Additionally, captured *Culicoides* specimens (excluding *Culicoides variipennis* Coquillett, 1901, which has widely been accepted as a non-vector organism) were subjected to real-time quantitative PCR (RT-qPCR) analysis in order to scan them for presence of BTV.

3.2 Materials and Methods

3.2.1 Specimen Capture and Sorting

In 2013 and 2014, the Sheep Farmers of Ontario were contacted in order to locate farmers willing to allow biting midge trapping on their livestock farms. Once a list of volunteers was compiled, farms were selected in a fashion that would maximize distribution of traps across southern Ontario. For 2013 sampling, five farms were selected for trapping between June 20th and August 30th. Volunteered farms were located in Stoney Creek, Mount Elgin, Ridgetown, Thamesville, and Sarnia. Addresses and GPS coordinates of these farms will be withheld in the interest of privacy. John Hock Miniature CDC light traps (Gainesville, FL) were placed at each farm on Thursday afternoon every two weeks and retrieved the day afterwards in the morning; this process was repeated for six trapping periods. This timeframe allowed for trapping during dusk and dawn, times at which biting midges are the most active. Light traps were outfitted with fine-mesh collection vessels to ensure biting midges could not escape once captured by the trap, as standard meshing is too wide to trap them successfully. Collection vessels were outfitted with a label, providing locality name and date of capture. Each trap was supplemented with a thermos containing 2 kg of dry ice. As the ice sublimated, it generated carbon dioxide which escaped through a small hole in the bottom of the thermos, acting as a chemical attractant for biting insects throughout the night. Upon trap retrieval, the collection vessels were sealed and detached from the light traps and placed into a cooler with dry ice to preserve the captured insects during the return trip to the laboratory. At the University, capture vessels were stored in the freezer until specimens could be sorted and tested.

In 2014, collection efforts were increased and expanded in an attempt to collect biting midges at more localities throughout the province. As a result of this decision, 10 sheep farms were sampled throughout the summer. Farms were allocated into two groups depending upon their distance from Brock University. The five farm localities in closest proximity (Niagara-on-

the-Lake, St. Ann's, Stoney Creek, Waterford, and Wellandport) to Brock University were labeled as 'Eastern Farms', while the remainder (Exeter, Lakeside, Lambton Shores, Oxford, Sarnia) were labeled as 'Western Farms.' Eastern and Western farms were sampled on alternating weeks: the first Eastern farm sample occurred on June 16-17, and the first Western farm sample occurred on June 23-24. This alternating sampling pattern was repeated until each set of farms was sampled 5 times. Traps established in 2014 were set and handled using the same trapping protocol as outlined above for 2013 insect sampling.

Prior to sorting, a chill plate was placed under a Leica MZ75 stereomicroscope, turned on, and left to cool until it reached -5°C. A 65 mm Petri dish was also placed on top of the chill plate and allowed to cool. A second chill plate was placed beside the microscope with a 20 mL scintillation vial on top of it and was also allowed to pre-cool. Traps were examined sequentially; subsamples of insects were removed from the collection vessel using a suction gun and placed into the 65 mm Petri dish, at which point the subsample was thoroughly examined for ceratopogonids. Biting midge specimens were removed and placed in the 20 mL scintillation vial for later storage in a -20°C freezer, and any remaining bycatch was disposed of. This process was repeated for each trap until all biting midge specimens were sorted and all bycatch was disposed of. This routine was performed on a weekly basis in order to empty the collection vessels in time for the next trapping session.

Culicoides specimens were sorted to subgenus level using the dichotomous key found in the *Manual of Nearctic Diptera, Vol. 1* (Downes and Wirth, 1981). In order to attain this level of taxonomic determination, dissections of the spermathecae of females were performed to visualize their structure. Additionally, *Culicoides* specimens of subgenus *Monoculicoides* were further identified to species level using the dichotomous key found in Holbrook *et al*. (2000).

Biting midges were stored in the -20°C freezer until further sorting was performed; this second round of sorting was done to create pools containing up to 100 similar Ceratopogonidae specimens. For set-up, a 65 mm Petri dish was placed on a chill plate and both were allowed to reach a temperature of -5°C. Additionally, an insulated cooling block outfitted with 12 holes for 2 mL roundbottom collection tubes had been frozen in a -20°C freezer beforehand. This cooling block was placed into a styrofoam container and surrounded tightly with ice in order to maintain a low temperature during sorting. Roundbottom vials were placed into the cooling block and used for biting midge pooling. Biting midges from a trap were subsampled from the respective 20 mL scintillation vial which was still in the freezer. This subsample was placed on the 65 mm Petri dish for examination under the stereomicroscope and biting midges of similar genus were sorted into the 2 mL roundbottom tubes; exceptions were made for any possible located BTV vector *Culicoides* (*Monoculicoides*) *variipennis sonorensis* and the closely related species *Culicoides* (*Monoculicoides*) *variipennis variipennis*, which were identified to species level and stored separately.

Numbers of each taxon were recorded. Once a 2 mL tube contained 100 of a similar taxon, it was closed and labeled with collection locality, collection date, number of specimens, and taxonomical classification. Each tube was assigned an identification code for further testing, comprised of a two-letter code representing locality, a shortened taxonomical identifier, and a number combination X-Y, where X indicates the trip number (1-5), and Y indicates the vial number if more than one was required to pool all specimens. For instance, a tube containing the second set of 100 *Forcipomyia* Meigen, 1818 specimens gathered at Stoney Creek on trip #2 was labeled as SC Forci 2-2 (trip 2, second pool of *Forcipomyia* specimens). After labeling, the collection tube was transferred into a labeled Nalgene 81-tube rack in the -20°C freezer. This

was performed for each sample until all Ceratopogonidae were sorted and pooled. If found, *Culicoides* specimens not belonging to subgenus *Monoculicoides* (referred to simply as *Culicoides* specimens for the remainder of this chapter), as well as *Culicoides sonorensis* specimens, were separated into their own species-level pools of up to 100 specimens for later subjection to RT-qPCR for virus testing. For each locality and date of collection, a maximum of 10 pools of specimens (1000 total specimens) were processed for virus detection.

3.2.2 Specimen Homogenization and RNA Extraction

A stock of diluent was created using the recipe found in Table 3.1. One mL of diluent was added to each sample; a small copper bead was also added to the tube using a pair of sterilized forceps. Samples were then placed in a Retcsh MM300 mixer mill and shaken for 2 minutes at a rate of 30 s/s to homogenize the specimens.

Table 3.1. Recipe for 100 mL of DMEM media used for specimen pool homogenization of biting midges. Volumes were subject to adjustment if differing amounts of DMEM media were required.

Ingredient name	Amount
1x Dulbecco's Modified Eagle Media (DMEM)	94.0 mL
Fetal Bovine Serum	5.0 mL
100X Penicillin Streptomycin	1.0 mL

Samples were centrifuged in a Thermo 21000 centrifuge at maximum speed (15,000 rpm) for 3 minutes. A pipettor was used to add 100 µL of each resulting supernatant to a microtube containing 250 µL of RLT lysis buffer. One volume (350 µL) of 70% ethanol was added to the sample and pipetted up and down to mix. The total volume of 700 µL was then added to a filtered spin column housed within a flow-through collection tube and centrifuged at maximum

speed for 3 minutes. Fluid that passed through the spin column was discarded, and 700 µL of RW1 buffer was added; this was centrifuged as per the previous step and the flow-through was discarded again. 500 µL of RPE buffer was added to the spin column, centrifuged, and discarded in the same fashion, followed by another 500 µL of RPE which was centrifuged for 6 minutes at maximum speed. Flow-through was disposed of, and the sample was centrifuged again for 1 minute at maximum speed to ensure all flow-through had been collected and discarded. The spin column was then placed in a sterile microcentrifuge tube, which was pre-labeled with the pool information code. At this point, 30 µL of RNase-free water was added to the column, and the sample was allowed to stand for 1 minute before being centrifuged at maximum speed for 3 minutes. The resulting RNA solution collected in the microcentrifuge tube was either subjected to RT-qPCR immediately or placed in the -20°C freezer for later use.

3.2.3 RT-qPCR Procedure

Tubes containing 2X QuantiTect Master Mix, QuantiTect RT Mix, RNase-free water, and VetMax/BTV_S1 primer/probe mix were placed in a styrofoam container filled with ice and used to make a reaction mix as per the recipe in Table 3.2.

Table 3.2. Recipe for BTV RT-qPCR reaction mix.

Name of Reactant	Amount Included
2X Quantitect Probe PCR Master Mix	200 µL
RNase-free Water	52 µL
VetMax™ Bluetongue Virus (BTV) Reagents	16 µL
Quantitect RT Mix	4 µL

Microcentrifuge tubes containing extracted RNA were placed in the ice. 6.4 µL of RNA from each sample was pipetted into separate low-profile optical wells. Additionally, 6.4 µL of VetMax™ Bluetongue Virus Control was pipetted into an additional well to serve as a positive control, and 6.4 µL of RNase-free water was pipetted into another well to act as a negative control. These were placed into a BioRad MYiQ™ thermocycler and heated to 95°C for 5 minutes, denaturing any double-stranded RNA contained within the sample. The optical wells were removed and placed back in the ice, at which point 13.6 µL of the reaction mix was added to each sample, including the positive and negative controls; these were pipetted up and down to adequately mix the contents. Once wells were filled with RNA and reaction mix, they were briefly centrifuged in a Thermo 21000 in order to remove any surface bubbles that would disrupt optical reading during the RT-qPCR process and to ensure all liquid was consolidated at the bottom of the wells. Wells were placed into a BioRad CFX Connect™ Real-Time PCR Detection System. The RT-qPCR thermocycler procedure outlined in Table 3.3 was used to assess presence of BTV.

Table 3.3. RT-qPCR procedure used for Bluetongue virus RNA detection. Each procedure involved 45 cycles of RNA amplification and subsequent detection after each cycle.

Step	Sample Temperature	Time Spent (mins: secs)
1. Reverse transcription	50.0˚C	30:00
2. Heat activation of DNA polymerase	95.0˚C	15:00
3. Denaturation	94.0˚C	00:15
4. Annealing/Extension	60.0˚C	01:00
	PLATE READ	
5. Repeat Process	**RETURN TO STEP 3 (44 times)**	

3.2.4 Verification of RNA Extraction

In order to ascertain that the RNA extraction procedure was being performed successfully and resulting in extracted RNA, VetMax™ Xeno™ Internal Positive Control RNA (containing

10,000 RNA copies/µL) was added to 10 samples of specimens which had already undergone homogenization. Internal positive control RNA was added directly into the RLT lysis buffer, allowing it to be present throughout the entirety of the RNA extraction procedure as outlined above. It was found that RNA was successfully extracted and detected using the outlined RT-qPCR methods. This test procedure indicates that any tested samples displaying no indication of BTV were doing so as a result of true absence of viral RNA, rather than because RNA simply was not being extracted properly (Figure 3.1).

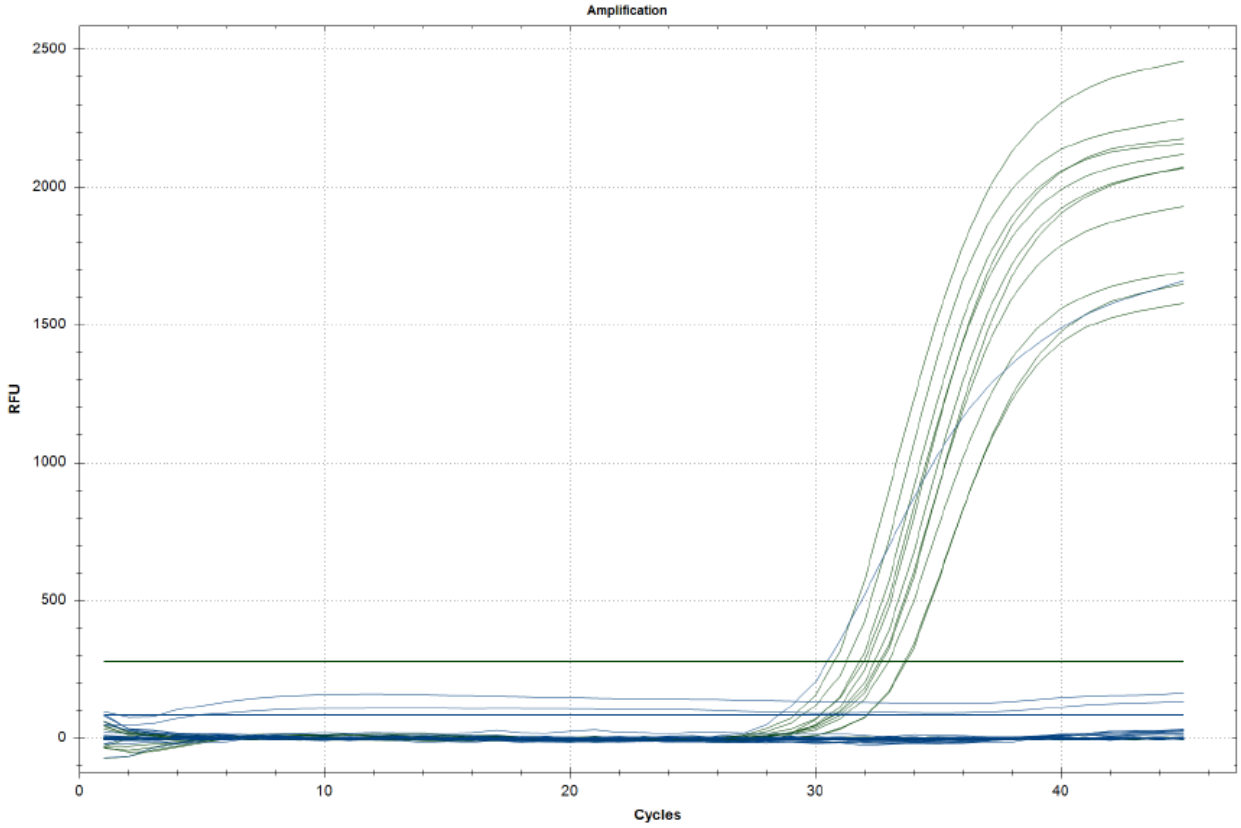

Figure 3.1. RT-qPCR result displaying presence of VetMax™ Xeno™ Internal Positive Control RNA (10,000 copies RNA/µL) in tested samples. Control RNA was added prior to the RNA extraction procedure. Presence of RNA in this graph, indicated by positive slopes beginning at approximately 30 cycles, shows that RNA is successfully extracted from processed samples using the methods described above. One control sample was also included and amplified, which contained inactive Bluetongue virus (BTV) gene sequences. Blue lines indicate FAM-490 fluorophore emission as a result of attachment to amplified Bluetongue virus gene sequences. Green lines indicate HEX fluorophore emission as a result of attachment to internal control RNA.

3.3 Results

3.3.1 Summer 2013 Sheep Farm Trapping Surveys

3.3.1.1 Discovery of Culicoides sonorensis *in Southern Ontario*

Taxonomic examination of captured specimens yielded the discovery of *Culicoides sonorensis* in the province, representing a new species record for Ontario. *C. sonorensis* was identified from three of five examined localities: Stoney Creek, Mount Elgin, and Sarnia (Table 3.4). A majority of the specimens were from Stoney Creek (n = 913), and 927 *C. sonorensis* specimens were found in total.

Table 3.4. Summary of Ceratopogonidae captured during 2013 sheep farm trapping. *Culicoides sonorensis* and *Culicoides variipennis* identified to species; other specimens identified to genus.

Trip 1 (Jun 20-21)	**Stoney Creek**	**Mount Elgin**	**Ridgetown**	**Thamesville**	**Sarnia**
Atrichopogon	-	-	1	-	-
C. sonorensis	357	-	-	-	-
C. variipennis	3	-	1	-	-
Culicoides	993	51	66	-	33
Forcipomyia	63	76	12	-	1
TOTAL	**1416**	**127**	**80**	**0**	**34**
Trip 2 (July 4-5)	**Stoney Creek**	**Mount Elgin**	**Ridgetown**	**Thamesville**	**Sarnia**
Atrichopogon	-	-	2	-	-
Bezzia	4	-	-	-	-
C. sonorensis	32	-	-	-	-
C. variipennis	2	-	5	-	-
Culicoides	631	339	375	3	109
Forcipomyia	1355	623	46	-	61
TOTAL	**2024**	**962**	**428**	**3**	**170**
Trip 3 (July 18-19)	**Stoney Creek**	**Mount Elgin**	**Ridgetown**	**Thamesville**	**Sarnia**
Atrichopogon	-	-	-	-	1
Bezzia	-	5	-	-	2
C. sonorensis	77	1	-	-	8
C. variipennis	1	76	-	-	6
Culicoides	12	38	5	-	14
Forcipomyia	2411	1150	8	-	995
Phaenobezzia	-	-	1	-	-
TOTAL	**2501**	**1270**	**14**	**0**	**1026**
Trip 4 (Aug 1-2)	**Stoney Creek**	**Mount Elgin**	**Ridgetown**	**Thamesville**	**Sarnia**
Atrichopogon	1	6	1	-	-
C. sonorensis	17	1	-	-	-
C. variipennis	-	2	-	-	2
Culicoides	2	33	34	2	36
Forcipomyia	9	7	-	-	658
TOTAL	**29**	**49**	**35**	**2**	**696**
Trip 5 (Aug 15-16)	**Stoney Creek**	**Mount Elgin**	**Ridgetown**	**Thamesville**	**Sarnia**
Atrichopogon	1	-	-	1	-
C. sonorensis	410	-	-	-	4
C. variipennis	7	-	-	-	7
Culicoides	79	4	2	1	26
Forcipomyia	149	-	7	-	408
TOTAL	**646**	**4**	**9**	**2**	**445**
Trip 6 (Aug 29-30)	**Stoney Creek**	**Mount Elgin**	**Ridgetown**	**Thamesville**	**Sarnia**
Atrichopogon	-	8	-	-	-
Bezzia	-	2	-	-	2
C. sonorensis	20	-	-	-	-
C. variipennis	-	1	-	-	1
Culicoides	52	30	-	-	4
Dasyhelea	-	1	-	-	-
Forcipomyia	-	61	-	-	49
TOTAL	**72**	**103**	**0**	**0**	**56**

A number of steps were taken in order to further verify the taxonomic identity of these specimens. The initial step involved direct visual comparison to verified, colony-reared *C. sonorensis* specimens donated by Dr. Tim Lysyk (Agriculture and Agri-Food Canada, Lethbridge, Alberta). Colony-reared specimens displayed key taxonomic features with high visual similarity to those captured at the Ontario sheep farms. Secondly, 10 specimens thought to be *C. sonorensis* from Stoney Creek were each sent to Dr. Lysyk and Dr. Art Borkent, dipterologists who focus their studies on Ceratopogonidae, for examination. Both of these contacts suggested that the initial taxonomic identification was correct.

Finally, molecular verification was performed, in which numerous gene regions of donated colony-reared *C. sonorensis* and field-captured *C. sonorensis* were compared. These regions were cytochrome oxidase subunit 1 (CO1), internal transcribed spacer 1 (ITS1), and elongation factor 1-alpha (EF1-α). All three gene regions were 100% identical between colony-reared *C. sonorensis* specimens and those found and identified in southern Ontario. Details of this process can be found in Chapter 4.

3.3.1.2 Assessment of BTV Presence in Collected 2013 Specimens

Pools of *Culicoides sonorensis* and non-*Monoculicoides Culicoides* specimens were processed for RT-qPCR using the protocol outlined in the Methods section. No pools of *Culicoides spp*. specimens (Table 3.5) or *Culicoides sonorensis* specimens (Table 3.6) collected in 2013 displayed positivity for presence of BTV; RT-qPCR graph results resembled those observed in Figure 3.2.

Table 3.5. List of 2013 *Culicoides* spp. pools, displaying locality of specimen collection, name of specimen pool, number of individuals in each specimen pool, and BTV status of each specimen pool.

Locality collected	Specimen pool code	Number of specimens	BTV detected?
Mount Elgin	ME Culi 1-1	51	No
Mount Elgin	ME Culi 2-1	100	No
Mount Elgin	ME Culi 2-2	100	No
Mount Elgin	ME Culi 2-3	100	No
Mount Elgin	ME Culi 2-9	39	No
Mount Elgin	ME Culi 3-1	38	No
Mount Elgin	ME Culi 4-1	33	No
Mount Elgin	ME Culi 5-1	4	No
Mount Elgin	ME Culi 6-1	30	No
Ridgetown	RT Culi 1-1	66	No
Ridgetown	RT Culi 2-1	100	No
Ridgetown	RT Culi 2-2	100	No
Ridgetown	RT Culi 2-3	100	No
Ridgetown	RT Culi 2-4	75	No
Ridgetown	RT Culi 3-1	5	No
Ridgetown	RT Culi 4-1	34	No
Ridgetown	RT Culi 5-1	2	No
Sarnia	SA Culi 1-1	33	No
Sarnia	SA Culi 2-1	100	No
Sarnia	SA Culi 2-2	9	No
Sarnia	SA Culi 3-1	14	No
Sarnia	SA Culi 4-1	36	No
Sarnia	SA Culi 5-1	26	No
Sarnia	SA Culi 6-1	4	No
Stoney Creek	SC Culi 1-1	100	No
Stoney Creek	SC Culi 1-2	100	No
Stoney Creek	SC Culi 1-3	100	No
Stoney Creek	SC Culi 1-4	100	No
Stoney Creek	SC Culi 1-5	100	No
Stoney Creek	SC Culi 1-6	100	No
Stoney Creek	SC Culi 1-7	100	No
Stoney Creek	SC Culi 1-8	100	No
Stoney Creek	SC Culi 1-9	100	No
Stoney Creek	SC Culi 1-10	93	No
Stoney Creek	SC Culi 2-1	100	No
Stoney Creek	SC Culi 2-2	100	No
Stoney Creek	SC Culi 2-3	100	No
Stoney Creek	SC Culi 2-4	100	No
Stoney Creek	SC Culi 2-5	100	No
Stoney Creek	SC Culi 2-6	100	No
Stoney Creek	SC Culi 2-7	31	No
Stoney Creek	SC Culi 3-1	12	No
Stoney Creek	SC Culi 4-1	2	No
Stoney Creek	SC Culi 5-1	79	No
Stoney Creek	SC Culi 6-1	52	No
Thamesville	TH Culi 2-1	3	No
Thamesville	TH Culi 4-1	2	No
Thamesville	TH Culi 5-1	1	No

Table 3.6. List of 2013 *Culicoides sonorensis* pools, displaying locality of specimen collection, name of specimen pool, number of individuals in each specimen pool, and BTV status of each specimen pool.

Locality Collected	Specimen Pool Code	Number of Specimens	BTV Detected?
Mount Elgin	ME Sono 3-1	1	No
Mount Elgin	ME Sono 4-1	1	No
Sarnia	SA Sono 3-1	8	No
Sarnia	SA Sono 5-1	4	No
Stoney Creek	SC Sono 1-1	100	No
Stoney Creek	SC Sono 1-2	100	No
Stoney Creek	SC Sono 1-3	100	No
Stoney Creek	SC Sono 1-4	57	No
Stoney Creek	SC Sono 2-1	32	No
Stoney Creek	SC Sono 3-1	77	No
Stoney Creek	SC Sono 4-1	17	No
Stoney Creek	SC Sono 5-1	100	No
Stoney Creek	SC Sono 5-2	100	No
Stoney Creek	SC Sono 5-3	100	No
Stoney Creek	SC Sono 5-4	100	No
Stoney Creek	SC Sono 5-5	10	No
Stoney Creek	SC Sono 6-1	20	No

3.3.2 Summer 2014 Sheep Farm Trapping Surveys

3.3.2.1 Recapture of Culicoides sonorensis *in Southern Ontario*

Specimens of *Culicoides sonorensis* were once again captured at sheep farms in 2014, with individuals collected at Sarnia, St. Ann's, Stoney Creek, Waterford, and Wellandport (Table 3.7, Table 3.8). Collection of the species in Stoney Creek and Sarnia (at the exact same farms) constitute recaptures in the same location. The other three localities are new records of *C. sonorensis* distribution in southern Ontario.

Table 3.7. Summary of Ceratopogonidae captured during 2014 sheep farm trapping at Eastern farms. *Culicoides sonorensis* and *Culicoides variipennis* identified to species; other specimens identified to genus.

Trip 1 (Jun 16-17)	**NOTL**	**St. Ann's**	**Stoney Creek**	**Waterford**	**Wellandport**
Atrichopogon	1	4	-	-	5
Bezzia	-	-	-	1	-
C. sonorensis	-	19	4	-	-
C. variipennis	-	159	1	-	-
Culicoides	866	16868	1639	25	136
Forcipomyia	-	92	128	984	24
TOTAL	**867**	**17142**	**1772**	**1010**	**165**
Trip 2 (July 2-3)	**NOTL**	**St. Ann's**	**Stoney Creek**	**Waterford**	**Wellandport**
C. sonorensis	-	25	6	-	1
C. variipennis	-	132	1	1	-
Culicoides	119	7532	80	162	352
Forcipomyia	6	464	32	1400	18
TOTAL	**125**	**8153**	**119**	**1563**	**371**
Trip 3 (July 14-15)	**NOTL**	**St. Ann's**	**Stoney Creek**	**Waterford**	**Wellandport**
Atrichopogon	-	-	-	-	1
C. sonorensis	-	-	15	-	1
C. variipennis	-	26	3	-	2
Culicoides	-	21	128	5	283
Forcipomyia	-	82	36	-	100
TOTAL	**0**	**129**	**182**	**5**	**387**
Trip 4 (July 28-29)	**NOTL**	**St. Ann's**	**Stoney Creek**	**Waterford**	**Wellandport**
Atrichopogon	1	-	-	-	-
C. sonorensis	-	-	70	-	2
C. variipennis	-	3	3	-	-
Culicoides	44	55	33	1	4
Forcipomyia	2	-	5	1	3
TOTAL	**47**	**58**	**111**	**2**	**9**
Trip 5 (Aug 11-12)	**NOTL**	**St. Ann's**	**Stoney Creek**	**Waterford**	**Wellandport**
Atrichopogon	-	-	-	1	-
C. sonorensis	-	-	-	-	-
C. variipennis	-	17	-	-	-
Culicoides	-	4	6	3	-
Forcipomyia	-	84	2	16	2
TOTAL	**0**	**105**	**8**	**20**	**2**

Table 3.8. Summary of Ceratopogonidae captured during 2014 sheep farm trapping at Western farms. *Culicoides sonorensis* and *Culicoides variipennis* identified to species; other specimens identified to genus.

Trip 1 (Jun 23-24)	**Exeter**	**Lakeside**	**Lambton Shores**	**Oxford**	**Sarnia**
C. variipennis	-	-	-	-	3
Culicoides	35	-	-	19	39
Forcipomyia	-	-	-	415	332
Palpomyia	-	-	-	1	
TOTAL	**35**	**0**	**0**	**435**	**374**
Trip 2 (July 7-8)	**Exeter**	**Lakeside**	**Lambton Shores**	**Oxford**	**Sarnia**
Atrichopogon	1	-	2	-	-
C. variipennis	-	1	-	-	-
Culicoides	22	29	149	1	20
Forcipomyia	-	14	225	-	1
TOTAL	**23**	**44**	**376**	**1**	**21**
Trip 3 (July 21-22)	**Exeter**	**Lakeside**	**Lambton Shores**	**Oxford**	**Sarnia**
Atrichopogon	-	2	-	-	-
Bezzia	-	2	-	-	3
C. sonorensis	-	-	-	-	1
C. variipennis	-	-	-	1	2
Culicoides	178	46	3	19	72
Forcipomyia	495	36	1	325	555
TOTAL	**673**	**86**	**4**	**345**	**633**
Trip 4 (Aug 5-6)	**Exeter**	**Lakeside**	**Lambton Shores**	**Oxford**	**Sarnia**
Atrichopogon	-	-	1	-	2
C. sonorensis	-	-	-	-	3
C. variipennis	-	1	-	-	1
Culicoides	7	63	8	3	388
Forcipomyia	78	18	-	114	14
TOTAL	**85**	**82**	**9**	**117**	**408**
Trip 5 (Aug 18-19)	**Exeter**	**Lakeside**	**Lambton Shores**	**Oxford**	**Sarnia**
Atrichopogon	1	1	1	-	-
C. variipennis	-	-	-	-	1
Culicoides	16	8	1	-	29
Forcipomyia	23	1	-	1	15
TOTAL	**40**	**10**	**2**	**1**	**45**

3.3.2.2 Assessment of BTV Presence in Collected 2014 Specimens

No pools of *Culicoides sonorensis* or other *Culicoides* spp. from either Eastern farm samples or Western farm samples displayed positivity for BTV (Table 3.9, Table 3.10, Table 3.11); RT-qPCR graph results resembled those observed in Figure 3.2.

Table 3.9. List of 2014 *Culicoides sonorensis* pools collected at Eastern farms and Western farms, displaying locality of specimen collection, name of specimen pool, number of individuals in each specimen pool, and BTV status of each specimen pool.

Locality Collected	Specimen Pool Code	Number of Specimens	BTV Detected?
Sarnia	SA Sono 3-1	1	No
Sarnia	SA Sono 4-1	3	No
St. Ann's	StA Sono 1-1	19	No
St. Ann's	StA Sono 2-1	25	No
Stoney Creek	SC Sono 1-1	4	No
Stoney Creek	SC Sono 2-1	6	No
Stoney Creek	SC Sono 3-1	15	No
Stoney Creek	SC Sono 4-1	70	No
Wellandport	WP Sono 2-1	1	No
Wellandport	WP Sono 3-1	1	No
Wellandport	WP Sono 4-1	2	No

Table 3.10. List of 2014 *Culicoides* spp. pools collected at Eastern farms, displaying locality of specimen collection, name of specimen pool, number of individuals in each specimen pool, and BTV status of each specimen pool.

Locality collected	Specimen pool code	Number of specimens	BTV detected?
Niagara-on-the-Lake	NOTL Culi 1-1	100	No
Niagara-on-the-Lake	NOTL Culi 1-2	100	No
Niagara-on-the-Lake	NOTL Culi 1-3	100	No
Niagara-on-the-Lake	NOTL Culi 1-4	100	No
Niagara-on-the-Lake	NOTL Culi 1-5	100	No
Niagara-on-the-Lake	NOTL Culi 1-6	100	No
Niagara-on-the-Lake	NOTL Culi 1-7	100	No
Niagara-on-the-Lake	NOTL Culi 1-8	100	No
Niagara-on-the-Lake	NOTL Culi 1-9	66	No
Niagara-on-the-Lake	NOTL Culi 2-1	100	No
Niagara-on-the-Lake	NOTL Culi 2-2	19	No
Niagara-on-the-Lake	NOTL Culi 4-1	44	No
St. Ann's	StA Culi 1-1	100	No
St. Ann's	StA Culi 1-2	100	No
St. Ann's	StA Culi 1-3	100	No
St. Ann's	StA Culi 1-4	100	No
St. Ann's	StA Culi 1-5	100	No
St. Ann's	StA Culi 1-6	100	No
St. Ann's	StA Culi 1-7	100	No
St. Ann's	StA Culi 1-8	100	No
St. Ann's	StA Culi 1-9	100	No
St. Ann's	StA Culi 1-10	100	No
St. Ann's	StA Culi 2-1	100	No
St. Ann's	StA Culi 2-2	100	No
St. Ann's	StA Culi 2-3	100	No
St. Ann's	StA Culi 2-4	100	No
St. Ann's	StA Culi 2-6	100	No
St. Ann's	StA Culi 2-7	100	No
St. Ann's	StA Culi 2-8	100	No
St. Ann's	StA Culi 2-9	100	No
St. Ann's	StA Culi 2-10	100	No
St. Ann's	StA Culi 3-1	21	No
St. Ann's	StA Culi 4-1	55	No
St. Ann's	StA Culi 5-1	4	No
Stoney Creek	SC Culi 1-1	100	No
Stoney Creek	SC Culi 1-1	100	No
Stoney Creek	SC Culi 1-1	100	No
Stoney Creek	SC Culi 1-1	100	No
Stoney Creek	SC Culi 1-1	100	No
Stoney Creek	SC Culi 1-1	100	No
Stoney Creek	SC Culi 1-1	100	No
Stoney Creek	SC Culi 1-1	100	No
Stoney Creek	SC Culi 1-1	100	No
Stoney Creek	SC Culi 1-1	100	No
Stoney Creek	SC Culi 1-1	100	No
Stoney Creek	SC Culi 2-1	80	No
Stoney Creek	SC Culi 3-1	100	No
Stoney Creek	SC Culi 3-2	28	No
Stoney Creek	SC Culi 4-1	33	No
Stoney Creek	SC Culi 5-1	6	No
Waterford	WA Culi 1-1	25	No

Waterford	WA Culi 2-1	100	No
Waterford	WA Culi 2-2	62	No
Waterford	WA Culi 3-1	5	No
Waterford	WA Culi 4-1	1	No
Waterford	WA Culi 5-1	3	No
Wellandport	WP Culi 1-1	100	No
Wellandport	WP Culi 1-2	36	No
Wellandport	WP Culi 2-1	100	No
Wellandport	WP Culi 2-2	100	No
Wellandport	WP Culi 2-3	100	No
Wellandport	WP Culi 2-4	52	No
Wellandport	WP Culi 3-1	100	No
Wellandport	WP Culi 3-2	100	No
Wellandport	WP Culi 3-3	83	No
Wellandport	WP Culi 4-1	4	No

Table 3.11. List of 2014 *Culicoides* spp. pools collected at Western farms, displaying locality of specimen collection, name of specimen pool, number of individuals in each specimen pool, and BTV status of each specimen pool.

Locality collected	Specimen pool code	Number of specimens	BTV detected?
Exeter	EX Culi 1-1	35	No
Exeter	EX Culi 2-1	29	No
Exeter	EX Culi 3-1	100	No
Exeter	EX Culi 3-2	78	No
Exeter	EX Culi 4-1	7	No
Exeter	EX Culi 5-1	16	No
Lakeside	LK Culi 2-1	29	No
Lakeside	LK Culi 3-1	46	No
Lakeside	LK Culi 4-1	63	No
Lakeside	LK Culi 5-1	8	No
Lambton Shores	LS Culi 2-1	100	No
Lambton Shores	LS Culi 2-2	49	No
Lambton Shores	LS Culi 3-1	3	No
Lambton Shores	LS Culi 4-1	8	No
Lambton Shores	LS Culi 5-1	1	No
Oxford	OX Culi 1-1	19	No
Oxford	OX Culi 2-1	1	No
Oxford	OX Culi 3-1	19	No
Oxford	OX Culi 4-1	3	No
Sarnia	SA Culi 1-1	39	No
Sarnia	SA Culi 2-1	20	No
Sarnia	SA Culi 3-1	72	No
Sarnia	SA Culi 4-1	100	No
Sarnia	SA Culi 4-2	100	No
Sarnia	SA Culi 4-3	100	No
Sarnia	SA Culi 4-4	88	No
Sarnia	SA Culi 5-1	29	No

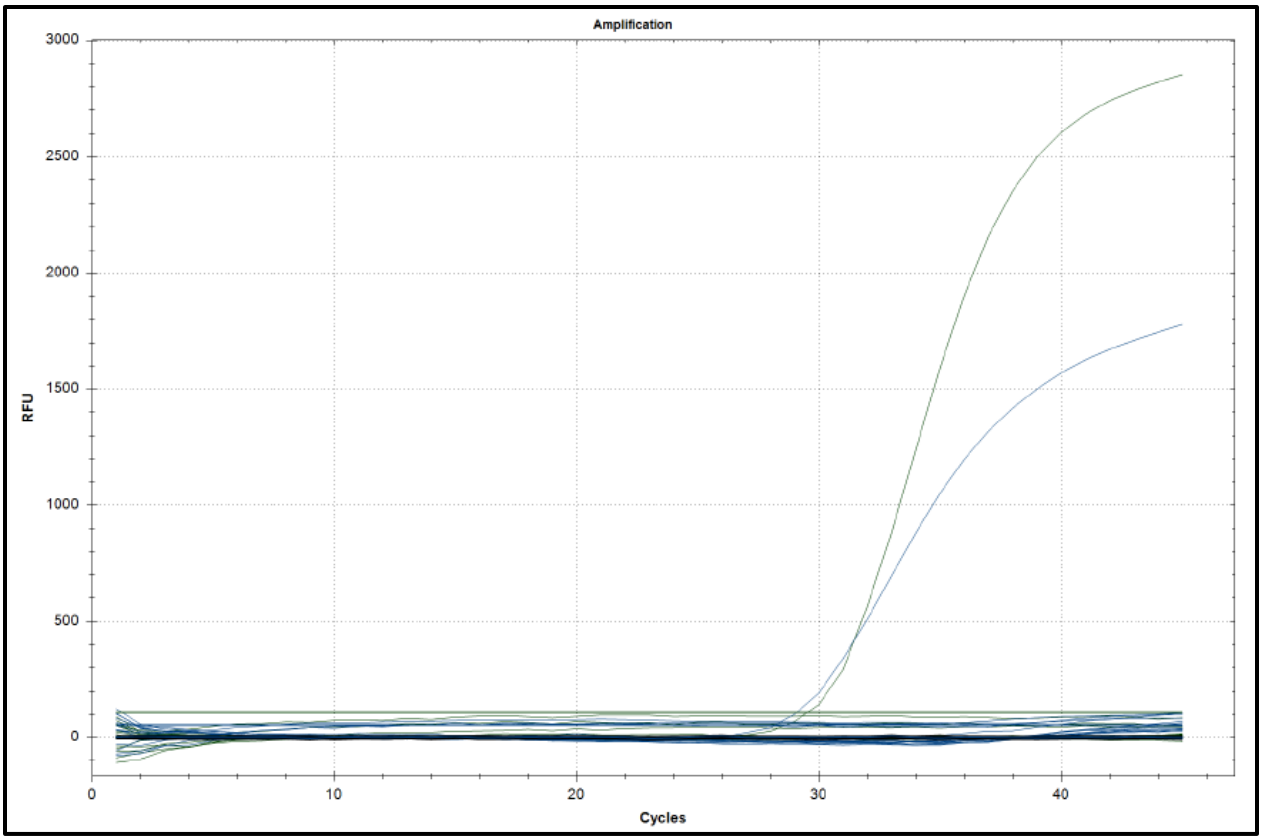

Figure 3.2. Example of graphed data from RT-qPCR analysis in which BTV was not detected. This graph is a visual representation of all conducted RT-qPCR analyses, which had similar results. Graph displays amplification of samples (RFU) on a per-cycle basis. Upward curves beginning at approximately the 30th cycle represent a positive control sample, displaying presence of BTV. Flat lines near the X axis throughout all cycles represent samples that did not contain BTV for amplification. Blue lines indicate FAM-490 fluorophore emission as a result of attachment to amplified Bluetongue virus gene sequences. Green lines indicate HEX fluorophore emission as a result of attachment to internal control RNA.

3.4 Discussion

3.4.1 Discovery of *Culicoides sonorensis* in Southern Ontario

Prior to this study, *Culicoides sonorensis* had not previously been located in any Canadian province outside of British Columbia and Alberta; as such, it was extremely surprising to find specimens of this species in multiple locations across southern Ontario. Additionally, *C. sonorensis* specimens were re-collected in similar localities in multiple years. This suggests that the species is able to successfully overwinter within the region, and it is quite possible that it has established itself as a permanent resident of the province.

The implications of this possible species establishment are quite serious. It is well known that *C. sonorensis* is the primary vector for Bluetongue virus in North America. This disease infects both wild and domestic ruminants, causing a number of symptoms that can eventually be fatal to the host (Tabachnick, 1996). Prior outbreaks of BTV have occurred in multiple regions of the world, with the most notable cases being in Spain and Portugal, and scattered outbreaks in multiple regions of North America, including the western Canadian provinces (See Chapter 2). The main vector of BTV in Spain and Portugal was *Culicoides imicola* Kieffer, 1913, a species of midge that is found throughout Africa, Europe, and parts of Asia; additionally, other resident species *C. obsoletus* (Meigen, 1818) and *C. pulicaris* (Linnaeus, 1758) have displayed potential BTV vector competence. The domestic livestock kept by farmers throughout these countries had not previously encountered the virus, and as such, were completely naïve to infection. Thousands of animals became infected and symptomatic, leading to virus containment via transport restriction, animal slaughter and, eventually, vaccination; overall, over 180,000 animals were killed during this incident (Pérez de Diego *et al*., 2013).

A series of 6 *C. sonorensis* specimens, originating from various localities of collection from both 2013 and 2014, have been set aside for deposition into the Canadian National Collection of Insects, Arachnids, and Nematodes for future reference.

3.4.2 Potential for BTV Infection in Ontario

Although the results of this study did not yield any biting midge specimens that were positive for BTV, it does not rule out the possibility that local midge populations are able to transmit the disease. After data collection for this project ended in 2014, three cattle displaying seropositivity for BTV were discovered in the Chatham-Kent region of Ontario during a province-wide serology survey in summer, 2015 (OIE, 2015). It was noted that all cattle were locally born and raised, suggesting that the animals were exposed to some form of the virus as a result of local interactions. Therefore, it is not unreasonable to conclude that local biting midges, likely specimens of *C. sonorensis*, are able to take up BTV and redistribute it to further mammalian hosts. Unfortunately, farms that were sampled in the Chatham-Kent region over the course of this study (Ridgetown, Thamesville) did not yield any *C. sonorensis* specimens, and any other *Culicoides* specimens that were sampled from these localities did not display any indication of BTV presence.

It is important to note that domestic livestock are not the only types of ungulates capable of contracting BTV. Various species of deer, including the widely-distributed white-tailed deer, may become afflicted with the virus if they are bitten by infected midges. Not only could this destabilize local deer populations, but infected deer could then infect other deer via further transmission through insect vectors. These aspects may allow the virus to become persistent in

Ontario, which may establish it as a significant threat to domestic and wild ungulates throughout the province. It may even be that local deer are becoming infected with BTV but going unnoticed, simply because deer are not as frequently observed by the public, and hunters who track deer may be unaware of BTV and its symptoms, making them unable to recognize an infected animal if they happen to encounter one in the wild.

It should also be noted that infections of epizootic hemorrhagic disease virus (EHDV) affecting white-tailed deer were recorded in the vicinity of London, Ontario in September of 2017 (CBC News, 2017). This virus is very closely related to BTV, to the point where they may cross-react if the incorrect detection tests are utilized. It is also vectored by *Culicoides sonorensis*; as such, it is possible that *C. sonorensis* populations exist near London, Ontario, and are transmitting the virus among local ungulate animals.

Overall, the newfound discovery of *Culicoides sonorensis* in Ontario alongside a small number of animals infected with BTV and EHDV in the last few years suggests that virus transmission is a distinct possibility within the province. Since many local ungulates are naïve to BTV and EHDV, it is likely that they will exhibit symptoms if they become infected by the bite of infected *C. sonorensis* midges. This may lead to outbreaks in the future. Thus, proper surveillance and precautionary measures need to be put in place to monitor for infections and track the distribution of *C. sonorensis* as it establishes itself across the province.

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Chapter 4: First Records of *Culicoides sonorensis* (Diptera: Ceratopogonidae), a Known Vector of Bluetongue Virus, in Southern Ontario

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4.1 Introduction

Certain members of genus *Culicoides* (Diptera: Ceratopogonidae), are vectors of diseases such as Epizootic Hemorrhagic Disease virus (EHDV) and Bluetongue virus (BTV), amongst a variety of others (Mellor et al. 2000, Brown et al. 2009, Purse et al. 2015). Although these viruses do not infect humans, they can cause harmful and potentially lethal symptoms in livestock such as sheep, goats, and cattle, as well as in wild ruminants, particularly deer (Tabachnick 1996).

The primary North American vector of BTV is *Culicoides sonorensis* Wirth & Jones 1957 (Carpenter et al., 2015), a member of the *Culicoides variipennis* species complex that also contains two other species native to North America: *Culicoides occidentalis* Wirth & Jones 1957 and *C. variipennis* (Coquillet, 1901) (Mellor et al. 2000). All species are known residents of Canada, with *C. variipennis* occurring in Ontario and British Columbia (Downes 1978), *C. occidentalis* in British Columbia (Downes 1978), and *C. sonorensis* in the western provinces of British Columbia and Alberta (Downes 1978, Lysyk and Dergousoff 2014). In the US, *C. variipennis* occurs east of the 100th meridian with relict populations in eastern Washington and western Montana, *C. occidentalis* has been recorded from California, Oregon, Nevada, New Mexico, and Texas, and *C. sonorensis* is fairly widespread west of the Mississippi river, occurring sporadically in the southeast. It is considered absent from the northeastern US, including the New England states that border Canada, as well as the northern states such as Pennsylvania, New York, and New Jersey (Holbrook et al., 2000)

Species distributions are not static, and there is always a possibility for range expansion of *Culicoides* species. During the summer of 2013, numerous specimens of *C. sonorensis* were

collected on sheep farms in Ontario. Morphological identifications were verified independently by Dr. Art Borkent and Dr. Tim Lysyk. Initially, we suspected that these represented temporary introductions of *C. sonorensis* capable of tolerating the summer conditions of southern Ontario. Therefore, we repeatedly sampled sites in 2013 and again in 2014 to determine if this species has established in Ontario. We believe that it should be treated as a threat to the livestock industry and its distribution should be monitored, as its capability as a viral vector not only imposes direct risk of infection by bluetongue virus, but can also impact transportation of livestock into and out of the province, increasing quarantine and transport fees.

Culicoides sonorensis can be distinguished fairly reliably from its close relative, *C. variipennis*, using structural traits described in taxonomic keys (Holbrook et al. 2000). Unfortunately, a high degree of taxonomic expertise is required to identify ceratopogonids and the number of suitably trained taxonomists in North America, especially Canada, is low. In addition, ceratopogonids are notably very small and some traits used for taxonomic identification can be difficult to observe without access to a research-quality microscope. One method of identification that can potentially be used to overcome this hurdle of taxonomical inexpertise is DNA barcoding, which has become widely recognized as a reliable tool for insect identifications. This technique only requires that a small portion of a specimen, such as an insect's leg, be used for the process; little to no taxonomic experience is required (Hebert et al. 2003). As a result, DNA barcoding could potentially enable an extensive surveillance program so that *C. sonorensis* can be monitored more effectively.

In order to successfully identify an organism with DNA barcoding, the specific gene sequences used for analysis must be available in accessible sequence databases. It is important to note that species identifications using DNA barcoding are only as reliable as the taxonomist-

verified specimens used to generate the DNA sequences. The barcoded gene region that is most often accepted for species identification is mitochondrial cytochrome oxidase subunit 1 (CO1). For this study, we selected three gene regions to amplify and sequence: cytochrome oxidase subunit 1 (CO1), internal transcribed spacer 1 (ITS1), and elongation factor 1 alpha (EF1α). This allowed us to compare the structure of each gene region to determine which would be the most suitable for molecular determination between *C. sonorensis* and *C. variipennis*.

CO1, a mitochondrial gene, has become an integral element for DNA barcoding (Hebert et al. 2003). ITS1 is a segment of DNA that occurs between two ribosomal structural RNA gene regions, known as 18S rDNA and 5.8S rDNA (Bower et al. 2008). Importantly, ITS1 displays a large amount of variation among species making it a good candidate for molecular identifications (Seifert 2009). EF1α is a relatively long gene (>1300 base pairs) used for translational elongation in a wide variety of organisms. EF1α is also subject to a high rate of evolution due to synonymous substitutions throughout the sequence (Simon et al. 2009). EF1α was originally used to determine phylogenetic relationships among genera and species of Lepidoptera, and was later established as a reliable marker for fungal species identification (Geiser et al. 2004).

4.2 Materials and Methods

Arthropods were collected at farms that were recruited by the Ontario Sheep Marketing Agency in southern Ontario, Canada. Farmers agreed to participate as long as the exact locations of their farms were kept confidential. In 2013, collections were made every other week at 5 sheep farms located in Mount Elgin, Ridgetown, Sarnia, Stoney Creek, and Thamesville. In 2014, efforts were doubled and collections were made at 10 sheep farms rather than five. New

traps were established in Huron, Lakeside, Lambton Shores, Niagara-on-the-Lake, St. Ann's, Waterford, and Wellandport; repeated collections were performed in the 2013 locations of Mount Elgin, Sarnia, and Stoney Creek. In total, 12 unique locations across Southern Ontario were sampled. Public Health Unit boundaries for sampled localities are as follows: Niagara (Niagara-on-the-Lake, Waterford, Wellandport), Hamilton (Stoney Creek, St. Ann's), Huron (Exeter), Oxford (Lakeside, Mount Elgin), Lambton (Sarnia, Lambton Shores), and Chatham-Kent (Ridgetown, Thamesville).

One John W. Hock CDC model 1312 miniature light trap (Gainesville, FL), powered by a 6-volt battery, was set at each farm. Light traps were equipped with ultraviolet light and were baited with carbon dioxide (dry ice) in an insulated container, in which a hole had been drilled to allow CO_2 dispersal. Traps were fitted with fine mesh collection nets, with perforations small enough to restrict arthropods (including Ceratopogonidae) from escaping. The light traps were set within 10 metres of each farm's livestock housing area, where sheep would be between dusk and dawn; ceratopogonids are known to be most active during these time periods (Downes and Wirth, 1981). Traps were set in the afternoon, left to operate overnight, and retrieved the following morning. Upon retrieval, collection nets were removed from the light traps, closed tightly with a drawstring, and placed in a cooler on ice for transport back to the lab at Brock University. At the laboratory, the collection nets were placed in a -20°C freezer to kill specimens for subsequent sorting, identification, and enumeration, all of which were performed on a chill plate. *Culicoides* specimens of subgenus *Monoculicoides* were further identified to species using dichotomous keys found in the *Manual of Nearctic Diptera, Vol. 1* (Downes and Wirth, 1981) and Holbrook et al. (2000). In order to identify *Culicoides* to subgenus level, dissection of female spermathecae was required. Collection data are reported according to Health Unit and

epidemiological week. Specimens of both *C. sonorensis* and *C. variipennis* were set aside for DNA extraction, molecular analysis, and genetic sequencing. Specimens of *C. sonorensis* from a lab-reared colony maintained at the USDA Arthropod-Borne Animal Disease Research Unit were provided by Dr. Lee Cohnstaedt (via Tim Lysyk, AAFC Lethbridge, AB) for comparative purposes.

DNA was extracted using the Qiagen QIAamp extraction kit (Hilden, Germany), following manufacturer's instructions. Gene-specific primers were used to amplify these regions using polymerase chain reaction (PCR) with a final 40 μL reaction mixture as follows: 5 μL DNA template, 1X Taq PLUS reaction buffer, 0.2 mM dNTPs, 0.3 μM each primer (0.4 μM for EF1α), and 0.5 unit of Taq PLUS (Norgen Biotek). Primers for EF1α were designed using the Primer BLAST software (NCBI), whereas primers for the other two gene regions were based on published CO1 (Hebert et al. 2003) and ITS1 (Li et al. 2002) primers (Table 4.1). PCR protocol details for the three gene regions are found in Table 4.2. PCR products were sent for Sanger sequencing at the Centre for Applied Genomics (Sick Kids, Toronto). ITS1 amplified from *C. variipennis* using H1/H2 primers failed to be directly sequenced as a result of enzyme slippage, so the primers were modified to contain restriction enzyme sites and the amplicon was inserted into plasmid pBARGEM7-2 (Pall and Brunelli 1993) to allow it to be sequenced. Sequence alignments were made using ClustalW (Larkin et al. 2007). The Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990) was used to compare sequences of *C. variipennis* from Ontario, *C. sonorensis* from Ontario and the colony-reared *C. sonorensis*, with sequences available in GenBank.

Table 4.1. Summary of primers used for amplification and sequencing of CO1, ITS1, and EF1α for *Culicoides sonorensis* and *Culicoides variipennis*.

Primer Name	Gene	Sequence (5'-3')	Information
LC01490	CO1	GGTCAACAAATCATAAAGATATTGG	Hebert et al. 2003
HC02198	CO1	TAAACTTCAGGGTGACCAAAAAATCA	
H1	ITS1	GTAGGTGAACCTGCGGAAGGATCAT	Li et al. 2002
H2	ITS1	GGTGCAATATGCGTTCAACA	
ITS1-resF	ITS1	AAT**GGATCC**GTAGGTGAACCTGC	**BamHI** restriction site
ITS1-resR	ITS1	AT**CTCGAG**GGTGCAATATGCGTT	**XhoI** restriction site
EF1α-F1	EF1α	ACTCTGGCAAGTCAACTACC	Custom designed
EF1α-R2	EF1α	ATAACACCGACAGCAACAG	

Table 4.2. Thermal cycler protocols for CO1, ITS1, and EF1a amplification.

	CO1		ITS1		EF1α	
	Temp.	Time	Temp.	Time	Temp.	Time
1. Denature	95°C	30 sec	95°C	5 min	95°C	5 min
2. 35 cycles	95°C	1 min	95°C	40 sec	95°C	30 sec
	40°C	1 min	50°C	40 sec	48°C	30 sec
	72°C	30 sec	72°C	1 min	72°C	90 sec
3. Final extension	72°C	5 min	72°C	3 min	72°C	5 min
4. Hold	4°C	-	4°C	-	4°C	-
Amplicon size	**658 bp**		**489 bp**		**1,264 bp**	

Average temperatures of collection dates were estimated by accessing climate data for weather station HAMILTON A on the Government of Canada Historical Climate Data website (Government of Canada, 2015). Station HAMILTON A is found at the approximate latitude of sampled localities, and also provides daily temperature averages for the region.

4.3 Results

Taxonomic identification of *Culicoides* (*Monoculicoides*) specimens revealed, as expected, that *C. variipennis* was present across southern Ontario, but so too was *C. sonorensis* (Figure 1). In 2013, a total of 927 *C. sonorensis* were collected from Stoney Creek, Mount Elgin, and Sarnia, and a total of 114 *C. variipennis* were collected from all localities except Thamesville. This finding marks the first discovery of *C. sonorensis* within the province of Ontario. Collections in 2014 yielded 99 *C. sonorensis* in two resampled locations (Sarnia and Stoney Creek), as well as 48 more specimens in two new localities (St. Ann's and Wellandport). *C. variipennis* was found once again in Mt. Elgin, Sarnia, and Stoney Creek (total, n = 16), but was also captured in Lakeside, St. Ann's, Waterford, and Wellandport (total, n = 342). The number of captured *C. sonorensis* and *C. variipennis* organized by locality and Health Unit is shown in Tables 4.3 to 4.5. Peak numbers of *C. sonorensis* were collected in Epiweek 33 (August 15-16) in 2013 and Epiweek 31 (July 28-29) in 2014. Peak populations for *C. variipennis* were in Epiweek 29 (July 18-19) in 2013 and Epiweek 25 (July 16-17) in 2014. Public Health Unit regions where *C. sonorensis* was collected are visualized in Figure 4.1.

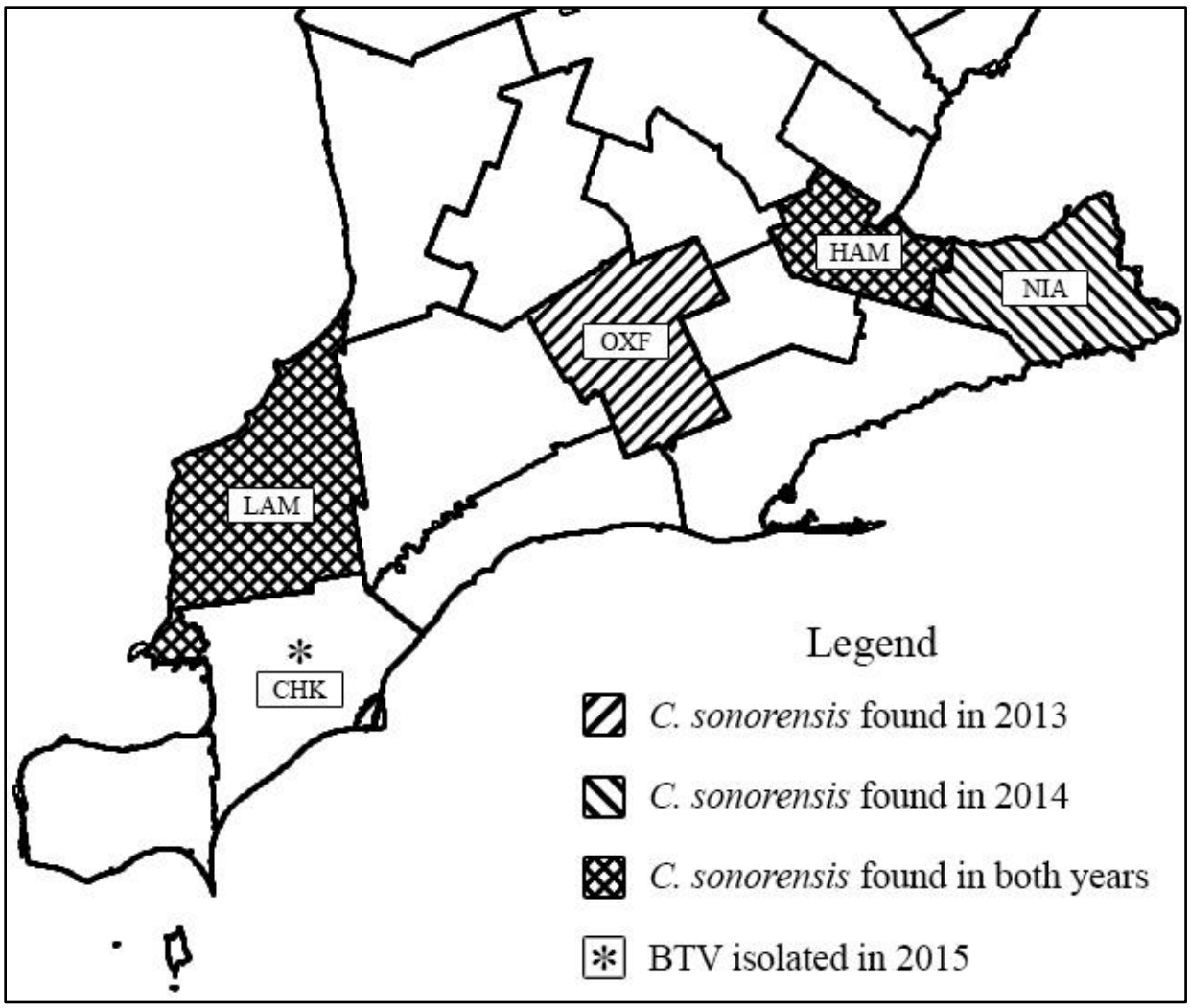

Figure 4.1. Distribution of *Culicoides sonorensis* in Health Units of Southern Ontario, Canada. Abbreviations are as follows: CHK, Chatham-Kent; HAM, Hamilton; LAM, Lambton; OXF, Oxford; NIA, Niagara.

Table 4.3. Number of *C. sonorensis* and *C. variipennis* captured in Stoney Creek (SC), Mount Elgin (ME), Sarnia (SR), Ridgetown (RT), and Thamesville (TH), Ontario in Summer 2013.

2013 (Hamilton, Oxford, Lambton, and Chatham Health Units)					
PHU	**Hamilton**	**Oxford**	**Lambton**	**Chatham**	
Locality	**SC**	**ME**	**SR**	**RT**	**TH**
C. sonorensis					
Jun 20-21	357	-	-	-	-
Jul 4-5	32	-	-	-	-
Jul 18-19	77	1	8	-	-
Aug 1-2	17	1	-	-	-
Aug 15-16	410	-	4	-	-
Aug 29-30	20	-	-	-	-
C. variipennis					
Jun 20-21	3	-	-	1	-
Jul 4-5	2	-	-	5	-
Jul 18-19	1	76	6	-	-
Aug 1-2	-	2	2	-	-
Aug 15-16	7	-	7	-	-
Aug 29-30	-	1	1	-	-

Table 4.4. Number of *C. sonorensis* and *C. variipennis* captured in Stoney Creek (SC), St. Ann's (StA), Niagara-on-the-Lake (NOTL), Waterford (WA), and Wellandport (WE), Ontario in Summer 2014.

2014 (Hamilton, Niagara Health Units)					
PHU	**Hamilton**		**Niagara**		
Locality	**SC**	**StA**	**NOTL**	**WA**	**WE**
C. sonorensis					
Jun 16-17	4	19	-	-	-
Jul 2-3	6	25	-	-	1
Jul 14-15	15	-	-	-	1
Jul 28-29	70	-	-	-	2
Aug 11-12	-	-	-	-	-
C. variipennis					
Jun 16-17	1	159	-	-	-
Jul 2-3	1	132	-	1	-
Jul 14-15	3	26	-	-	2
Jul 28-29	3	3	-	-	-
Aug 11-12	-	17	-	-	-

Table 4.5. Number of *C. sonorensis* and *C. variipennis* captured in Exeter (EX), Lambton Shores (LS), Sarnia (SR), Lakeside (LK), and Mount Elgin (ME), Ontario in Summer 2014.

2014 (Huron, Lambton, Oxford Health Units)					
PHU	**Huron**	**Lambton**		**Oxford**	
Locality	**EX**	**LS**	**SR**	**LK**	**ME**
C. sonorensis					
Jun 23-24	-	-	-	-	-
Jul 7-8	-	-	-	-	-
Jul 21-22	-	-	1	-	-
Aug 5-6	-	-	3	-	-
Aug 18-19	-	-	-	-	-
C. variipennis					
Jun 23-24	-	-	3	-	-
Jul 7-8	-	-	-	1	-
Jul 21-22	-	-	2	-	1
Aug 5-6	-	-	1	1	-
Aug 18-19	-	-	1	-	-

Sequences that we have generated for CO1, ITS1, and EF1α for *C. sonorensis* and *C. variipennis* have been deposited into GenBank with Accession numbers KP310072 to KP310110. A CO1 gene sequence for *C. sonorensis* was found in GenBank (Accession number JF870510) whereas there were no CO1 sequence data for *C. variipennis*. CO1 sequences of our wild-caught *C. sonorensis* specimens were 100% identical to verified colony specimens of *C. sonorensis*. However, CO1 sequences from our *C. sonorensis* and *C. variipennis* specimens also displayed 100% similarity. The GenBank sequence was only 97% similar to our *C. sonorensis* specimens (Supp. 1).

For ITS1, wild-caught *C. sonorensis* specimens (n=5) from Ontario and colony-reared *C. sonorensis* specimens (n=5) were identical. ITS1 sequences from wild-caught *C. sonorensis* and wild-caught *C. variipennis* specimens (n=5) displayed ~98% similarity, with only a small number of nucleotide differences and gaps in the alignment. An ITS1 sequence for *C. sonorensis*

was not available in GenBank, although there was a sequence for *C. variipennis* (Accession number U48380) that did not line up with our wild-caught specimens (Supp. 2).

The *C. sonorensis* EF1α gene sequence was found in GenBank (Accession number AY752802), but our specimens only showed 98% similarity and 90% query coverage with it. A large number of unmatched nucleotides throughout the sequence alignment involved thymine residues. The GenBank *C. sonorensis* EF1α sequence was determined using mRNA rather than DNA, and thus, intron sequences were lacking. For EF1α, our *C. sonorensis* and *C. variipennis* sequences displayed 99% similarity, with 18 nucleotide differences between the two species (Supp. 3). Of these differences, 9 occurred within the introns of the gene. Intron 1, located at *C. sonorensis* nucleotides 832-895 and *C. variipennis* nucleotides 834-897, contained seven different nucleotide substitutions. Intron 2 at *C. sonorensis* nucleotides 1138-1212 and *C. variipennis* nucleotides 1140-1214 contained two (Figure 2).

```
EF1a Intron 1:
CS  gtaagtagtttgaaataatcatttttgcctttacaaaattattgtaataatatttcatttatag  64
CV  ..........a......c.........ca.............t..c......t........  64

EF1a Intron 2:
  CS  attgatatgataaacagttaattttgatgcttactttataaattattttacagtggaaga  60
  CV  ........a................t.................................  60
```

Figure 4.2. Two elongation factor 1-alpha (EF1α) introns from *Culicoides sonorensis* (CS) and *Culicoides variipennis* (CV). Periods denote similarities in the sequence, while letters show differences between the sequences of the two species.

4.4 Discussion

During 2015, the Canadian Food Inspection Agency (CFIA) discovered one cattle farm in Chatham-Kent, Ontario where three animals were found to be seropositive for BTV-13 (OIE 2015). This is the first recorded case of BTV in the province and in Canada outside the

Okanagan Valley of British Columbia. The seropositive animals had not been imported into Canada, and had remained in Chatham-Kent for their entire lives. This suggests that the animals were exposed to BTV as a result of local interaction with infected biting midges. Based on our findings, we hypothesize that the vector is most likely *C. sonorensis*.

Over the course of this study, sampling efforts in 2013 included routine insect trapping at farms in Ridgetown and Thamesville, two localities situated within the Chatham-Kent county. Interestingly, no *C. sonorensis* were found at either farm, although 6 specimens of *C. variipennis* were collected in Ridgetown.

Collection data from 2013 and 2014 suggest that populations of *C. variipennis* and *C. sonorensis* may peak at different times in the season. In the Hamilton region in 2013, a large peak of *C. sonorensis* occurred in Epiweek 25 (n=357; June 20-21, Avg Temp = 17.8°C) and an even larger peak in Epiweek 33 (n=410; August 15-16, Avg Temp = 16.4°C). In contrast, the largest number of *C. variipennis* was found during Epiweek 29 (n= 76; July 18-19, Avg Temp = 27.6°C) in Oxford County; low numbers of this species were found in all other regions throughout the season. In 2014, peak numbers of *C. sonorensis* were collected during Epiweek 31 (n=70; July 28-29, Avg Temp = 15.9°C) from farms located in the Niagara region, while the highest numbers of *C. variipennis* were collected from the same region during Epiweek 25 (n=159; June 16-17, Avg Temp = 21.4°C). This is important base-line information for distribution patterns of the two species.

A CO1 sequence was available in GenBank for *C. sonorensis* (Accession number JF870510; submitted April 2011), but not for *C. variipennis*. This seemed unusual, as *C. variipennis* is known to be widespread throughout North America and, as such, should be

relatively easy to collect and sequence. The wild-caught specimens of *C. sonorensis* from Ontario shared 97% of their CO1 genome with JF870510; this was the top match for every specimen that was successfully sequenced (n=5). Fortunately, we also sequenced taxonomically-verified *C. variipennis* and colony-reared *C. sonorensis*. Instead of solving the problem, we discovered that our new sequences were more closely related to one another and to our wild caught *C. sonorensis* than any of the sequences were to JF870510 (Supp. 1). This suggests two possibilities: either *C. sonorensis* is well represented by JF870510 (based on a California specimen) and the species has accumulated mutations as it has expanded its range across North America, or JF870510 is based on a misidentification and belongs to a different member of the *C. variipennis* species group.

For ITS1, there was a single *C. variipennis* sequence (U48380; colony reared, collection data not supplied) but no *C. sonorensis* sequence in GenBank. The specimens from Ontario that keyed out as *C. sonorensis* showed the highest sequence similarity (at >94%) to U48380 (Supp. 2). It is important to note that with no *C. sonorensis* ITS1 sequences in GenBank, our specimens might simply have been misidentified as *C. variipennis* by non-taxonomists having no reason to suspect otherwise. Had such a molecular identification been accepted, the range expansion of *C. sonorensis*, a potentially dangerous livestock pest, would have gone unnoticed. Importantly, for the ITS1 region, colony-reared *C. sonorensis* sequences were identical to those of our wild-caught *C. sonorensis* from Ontario; both can be reliably differentiated from *C. variipennis* from Ontario based on a number of insertions and deletions.

Alignments between the GenBank sequence for *C. sonorensis* EF1α (Accession number AY752802; submitted September 2004) and EF1α from our wild-caught *C. sonorensis* specimens displayed two large gaps because AY752802 was obtained from messenger RNA

rather than DNA (Supp. 3). EF1α sequences from wild-caught *C. sonorensis* showed 100% similarity with those from colony-raised *C. sonorensis*. Interestingly, the introns of the EF1α gene contained 50% of the genetic differences between the *C. sonorensis* and *C. variipennis*, with 7 differences in Intron 1, and two in Intron 2 (Fig. 2). The EF1α intron sequences will likely be quite useful for population-level studies in the future.

Culicoides sonorensis is the primary vector for bluetongue virus in North America, and is also capable of transmitting other harmful diseases such as African horse sickness virus (Mellor et al. 2000). Although much information for differentiating this species from other members of the *C. variipennis* complex was provided by electrophoretic isozyme analysis (Tabachnick 1996), modern molecular identification has been drifting towards comparisons of gene structure, such as the three regions used in this study. Due to its importance within the field of veterinary entomology, we suggest that priority be placed on gathering as much gene sequence information as possible about *C. sonorensis* to maximize the accuracy of molecular identification. Documenting gene sequences of *Culicoides occidentalis* is also encouraged. If such reference sequences are not found within publicly available databases, molecular misidentifications may easily occur. These species need to be monitored throughout North America to map further range expansions; increasing the amount of molecular sequence data available for the *C. variipennis* species complex as a whole is a relatively simple way to achieve this goal.

Culicoides sonorensis was only previously recorded in the western Canadian provinces of British Columbia (Downes 1978) and Alberta (Lysyk and Degousoff 2014), as well as in the United States, ranging as far north as North Dakota (Schmidtmann et al. 2011) and Montana (Zuliani et al. 2015). Prior to this study, the species had not been found in Ontario. As such, the collection of *C. sonorensis* within the province is a significant finding, as it was originally

thought that the species was absent from this area. Collection of *C. sonorensis* within Ontario over the course of this study, particularly in the same localities over two years, implies that at least some individuals are able to successfully overwinter in the southern regions of the province. Collection patterns appear to indicate that populations of *C. sonorensis* currently occur sporadically in Ontario, mimicking distribution trends observed in the southern latitudes of eastern North America (Schmidtmann et al. 1998).

With the species being found at latitudes higher than those of southern Ontario, these trends suggest that *C. sonorensis* expansion may not be restricted exclusively by overall climatic patterns, but also by local factors that favour or limit the establishment of a new population, resulting in patchy distributions. In the last few years, winters in the southern regions of Ontario have become notably less harsh, exhibiting warmer temperatures between November and April. Seasons such as these may help to facilitate establishment and persistence of *C. sonorensis* populations within Ontario, allowing them to reach areas with favourable conditions for development such as livestock farmland. Furthermore, if bluetongue virus is truly present, shared habitats between infected animals and *C. sonorensis* populations could result in significant infection of livestock and affect transportation of animals.

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Chapter 5: An Analysis of Biting Midges (Diptera: Ceratopogonidae) Collected by the Brock University Rothamsted Trap in Relation to Local Climate Patterns

5.1 Introduction

In the Canadian climate, many insects remain in states of hibernation or as aquatic larval phases throughout the colder winter months. However, during the remaining seasons, adult insects may be present in high numbers, such that they are often perceived as pests. Springtime in Canada yields vernal pools; these are temporary water bodies created by snowmelt, which can serve as aquatic habitats for various insect taxa for a limited amount of time (Colburn *et al*., 2007). Vernal pools also lack many natural predators for particular insects, making them ideal as areas for development of aquatic and semi-aquatic insects during their immature life stages. In addition to snowmelt, rainwater beginning in the spring and continuing through the summer can establish or support these types of environments, allowing insects to persist within them for longer or to establish new populations in recently-developed pools.

Numerous families of biting Diptera (true flies) exhibit life cycles with aquatic or semi-aquatic larval stages. Such families include many of those found in the dipteran suborder Nematocera, commonly known as the long-horned flies (Downes and Wirth, 1981). Medically significant and widely-recognized families are contained within Nematocera, such as Culicidae (the mosquitoes) and Simuliidae (the black flies), but the family Ceratopogonidae (the biting midges) is much less recognized by the public and also studied significantly less, despite the fact that specific members of this family act as vectors of harmful veterinary diseases. The genus *Culicoides* Latreille, 1809, for example, contains multiple species that are capable of transmitting livestock-affecting viruses, such as bluetongue virus (BTV) and epizootic haemorrhagic disease virus (EHDV). These diseases alone have been responsible for sickness and death in thousands of ungulate animals worldwide (Pérez de Diego *et al*., 2013).

Biting midges have been the primary identified vector in various viral outbreaks in multiple continents. For example, *Culicoides imicola* Kieffer, 1913 was implicated as the primary vector during outbreaks of bluetongue virus in Europe, specifically in the countries of Spain and Portugal in the late 1950's (Wilson and Mellor, 2009). The same species also vectored African horse sickness virus, a close relative of BTV, throughout Spain (Mellor, 1993). Further outbreaks occurred in other locations, such as the Iberian Peninsula, Belgium, and Greece, in which *Culicoides* biting midges were found to be the vectors (Wilson and Mellor, 2009).

Prior to additional BTV outbreaks in Europe in 2006, pest species such as aphids had been monitored using Rothamsted traps, which were later used further for monitoring *Culicoides* populations (Fassotte *et al*., 2008). With a total height of approximately 12 metres, Rothamsted traps consist of a rectangular base containing a sample bottle filled (usually containing 70% ethyl alcohol as a killing agent). A tall and hollow cylindrical column protrudes from the top of the base. The trap is outfitted with a strong electric fan used to generate suction force, which pulls air downward through the cylindrical column and into an expanded chamber within the base, which reduces the air speed and prevents captured insects from colliding roughly with the sides of the chamber to reduce specimen damage (Fassotte *et al*., 2008; Figure 5.1). As a result, any insects which happen to pass just overtop of the column are drawn into it and downwards, entering the bottle of ethanol where they are killed for later identification and analysis. The height of the trap is deliberate, as it allows for continuous sampling of a specific layer known as the aerial plankton. Within this layer, small organisms, such as insects that are only capable of fairly weak flight, are carried by wind currents (Fassotte *et al*., 2008). As such, the Rothamsted trap is able to collect consistent and unbiased samples of insects that are commonly found in the region as they pass overhead.

Figure 5.1. The Brock University Rothamsted Trap. Insects are captured at the top of the trap via suction and brought down into a collection bottle, housed within the rectangular base.

It is then possible to identify the specimens captured in the Rothamsted trap and use these data to properly assess local populations of potential vector insects, including Ceratopogonidae. Due to the consistent sampling effort of the Rothamsted, and the unbiased nature of its sampling method, insects captured in the trap provide a relatively accurate measure of biting midge diversity within the area on a daily basis. This information can be used to analyze the phenological patterns of medically significant insects and determine when large numbers of vectors are present. Such information is important for livestock producers, allowing them to assess when preventive measures should be put into place in order to limit contact time between their livestock animals and vector insects (such as *Culicoides* species), potentially reducing disease occurrence in their flocks or herds and reducing the likelihood of large outbreaks occurring. In addition, this information can also be cross-referenced with meteorological records to determine the types of conditions that favour high populations of vector organisms, which in turn may allow one to recognize when to enforce or increase animal protection from insect bites to reduce the chances of disease transmission.

5.1.1 Ceratopogonidae Genera of Particular Interest

Preliminary observations from Rothamsted sampling in the summer season of 2012 suggested high abundances of three Ceratopogonidae genera in the local fauna: *Bezzia* Kieffer, 1899, *Culicoides*, and *Probezzia* Kieffer, 1906. As such, these genera were the primary focus of abundance data analysis in Rothamsted samples collected from 2013 to 2017.

5.1.1.1 Bezzia

Despite being quite common in aquatic systems throughout North America, details regarding *Bezzia* biology and behaviour appear to be sparse in literature. Larvae are elongate and legless, progressing through four instars in moistened or aquatic habitats such as rivers and lakes, where they are suspected to prey upon various microorganisms (Downes and Wirth, 1981; Ronderos and Spinelli, 2009). Larvae have also been recorded as predators of aquatic or subaquatic dipteran eggs, including those of shore flies (Diptera: Ephydridae; Dow and Turner, Jr., 1976), and some are suspected to be cannibalistic (Wirth, 1983).

After pupation, adults feed upon insects and other small invertebrates, though it is mentioned multiple times in literature that one of their primary sources of nutrition is the haemolymph of non-biting midges (Diptera: Chironomidae) and mayflies (Order Ephemeroptera), which they attack mid-flight (Downes, 1978; Wirth, 1983; Ronderos and Spinelli, 2009). Female *Bezzia* have been observed preying on chironomid flies during mating swarms, and also exhibit sexual cannibalism, where the females consume the haemolymph of their male copulatory partners during mating (Downes, 1978; Wirth, 1983).

Bezzia habits and biology establish them as low-importance insects with respect to medical and veterinary diseases.

5.1.1.2 Culicoides

Since *Culicoides* species are widely recognized as disease vectors, their biology is fairly well understood as a result of their medical and veterinary significance. Eggs are laid on a

substrate in moist environments or near the edges of water bodies, where larvae hatch and develop through four larval instars. *Culicoides* may overwinter as larvae, and larvae have also displayed rapid progression through all four instar stages as a result of high ambient temperature. After pupation, pupal forms may individually float at the top of a moist area or body of water, or may adhere to material for support. After emergence, adults often survive for 10-20 days (Mellor *et al*., 2000).

Approximately 96% of all 1400 recognized species of *Culicoides* have females that take a blood meal for egg development, and a majority of organisms fed upon by *Culicoides* are birds or mammals. As a result of their blood-feeding habits, various species of *Culicoides* are responsible for transmission of numerous viral diseases worldwide, including BTV and EHDV, as mentioned above (Mellor *et al*., 2000). Additionally, their small size and weak capability for flight makes them susceptible to dispersion by wind, at least to some degree (Ducheyne *et al*., 2007; Eagles *et al*., 2011; Sedda *et al*, 2012). This combination of factors allows them to function as significant vectors of disease, capable of introducing viruses and causing outbreaks in new populations of animals, such as livestock and wildlife ruminants. As such, *Culicoides* is a very significant genus and it is important to understand the distribution and habits of disease-carrying species.

5.1.1.3 Probezzia

Members of the genus *Probezzia* have some similarities to *Bezzia*. *Probezzia* are rather large in size, relative to other genera of Ceratopogonidae, and tend to have prominent markings and patterns, particularly on their femora and tibiae. Common in standing and flowing water

bodies throughout North America, *Probezzia* larvae progress through four instar stages at which point they pupate in the water body. Interestingly, pupal forms of some *Probezzia* species have displayed the ability adhere to the stems of emergent plants via the use of glandular discs on the abdominal sternites which secrete a glue-like substance. Presumably, this behaviour prevents the pupae from being displaced by strong waves or flooding events. The pupae are also somewhat motile, and are able to re-establish their attachment if they become disturbed or dislodged (Wirth, 1971; Wirth, 1994).

Adult *Probezzia* primarily feed on small insects with soft exoskeletons, extracting haemolymph required for nutrition and egg development; examples of such insects include Chironomidae and Ephemeroptera (Downes, 1978). *Probezzia* species have also been observed exhibiting sexual cannibalism. While a male and female *Probezzia* are mating, the female may consume the haemolymph of her partner until the male is dead. The body of the male eventually detaches and falls to the ground, though his genitalia often remain attached to those of the female (Wirth, 1971; Downes, 1978; Wirth, 1994).

The habits of *Probezzia* species establishes them as incapable of vectoring human and livestock diseases, and therefore they are not of medical and veterinary importance.

5.2 Materials and Methods

5.2.1 Specimen Collection and Analysis

The Brock University Rothamsted trap is located at the Decew Falls water treatment plant at 2700 Decew Road in St. Catharines, Ontario, Canada. It was constructed in 2012 and has

been fully operational in the summer seasons since its establishment. The trap was operational from the late spring to the early fall seasons on a yearly basis; for this project, data from operational periods of 2013-2017 were used. During this time, the Rothamsted trap collected insects in 500 mL plastic wide-mouth Nalgene bottles filled approximately half-way with 70% ethyl alcohol; bottles were not filled completely to account for possible rain that may accumulate during storms or rainfall, which may flood the sample and result in lost insect specimens as a result. The ethanol bottle was removed and replaced with a new sample bottle every day at approximately 10:15 am, with the exception of weekends when collections ran from 10:15 am on Friday until 10:15 am on Monday, at which point daily collections were resumed. For analysis of sample contents, each sample was emptied into a clear glass sorting dish and examined thoroughly using a Leica MZ75 microscope. For each sample bottle, all Ceratopogonidae were removed, identified to genus level, tallied by genus, and placed into a 20 mL scintillation vial. Specimens were identified to genus using Ceratopogonidae dichotomous keys found in the *Manual of Nearctic Diptera, Vol. 1* (Downes and Wirth, 1981), and the *Manual of Central American Diptera, Vol. 1* (Borkent *et al*., 2009); no dissections were required as external physical features allowed identification to genus level. Vials were labelled with collection information, which included date(s) of capture and the number of Ceratopogonidae found in the sample. Many specimens collected by the Rothamsted trap were also later used as photographic subjects for a visual dichotomous key to the Nearctic genera of Ceratopogonidae (See Chapter 6).

All data were recorded in Microsoft Excel for later analysis. Each sample was tallied separately, with weekday samples encompassing one 24-hour time period (10:15 am on one day to 10:15 am the following day) and weekend samples consisting of one 72-hour time period

(10:15 am on Friday to 10:15 am the following Monday). In order to eliminate bias due to differing sample collection patterns between weekdays and weekends, all data were organized on a weekly basis. For instance, one week of data encapsulated a timespan of 10:15 a.m. Wednesday to 10:15 a.m. the following Wednesday, accounting for seven days of capture in each subset. This method of analysis was used to compensate for the inability to record and observe individual daily captures in the weekend samples. In order to visualize demographic patterns of captured Ceratopogonidae genera, bar graphs were generated from the resulting data. Graphs specifically displayed population data pertaining to the three most abundant genera throughout the five years of data collection. Additionally, redundancy analysis (RDA) was used to evaluate the validity of apparent trends in the data to determine their significance, allowing insight into the effects of climate variables on Ceratopogonidae populations, abundance, and diversity.

5.2.2 Variables for Meteorological Analysis

Climate data were acquired from Environment and Climate Change Canada (weather.gc.ca). Canada Weather Stats provides easily accessible climate data for multiple factors for localities throughout the country, providing average values for each variable at specific timestamps based on measurements recorded by all weather stations within that locality. For example, the average temperature of St. Catharines on a specific day in the summer, as reported by Canada Weather Stats, would be derived from all weather stations reporting the temperature on that specific day. Averaged data provides a better estimate of the overall

conditions of the locality, which is particularly useful when there are no specific weather stations near to the Rothamsted trap from which to gather data.

Variables that were analyzed for the purpose of this study include daily recordings of Average Temperature (˚C), Average Relative Humidity (%), Average Wind Speed (km/h; kilometres per hour), Average Pressure (kPa; kilopascals), and Precipitation (mm).

Precipitation data were not readily available for St. Catharines during the months when the Rothamsted trap was active and collecting insects. Only one station within St. Catharines, located at Brock University, reported consistent precipitation data; however, these data were only collected between November and April, presumably to assess the amount of snowfall to make decisions about possible class cancellations. As a result, precipitation data were tabulated from the proximal locality of Vineland, approximately 15 kilometres away from St. Catharines. Though this shift in data collection for precipitation is not ideal, precipitation data were collected year-round for this location, whereas other localities near St. Catharines displayed spotty data recording and inconsistent measurements. Vineland and St. Catharines encounter similar rain patterns due to their proximity to one another, and so using data from Vineland seemed like the most reasonable option to gather values for this particular variable.

5.2.3 Assessment of Meteorological Factors Affecting Biting Midge Activity

In order to properly use RDA, data were organized in a particular way, depending on the variable being analyzed. Genus tally data were analyzed on a weekly basis, as described above, resulting in a total of 107 data collection timeframes. Climate data for all five meteorological variables were averaged for each genus tally timeframe, providing an overall value for the entire

week ranging from Wednesday to the following Tuesday. All data was organized into two matrices to prepare them for RDA: a genus matrix, in which all genus tally data from 2013 to 2017 were organized by week; and a climate matrix, which contained data for all five meteorological variables under analysis. In order to optimize analysis via RDA, any data with decimal values were rounded to one decimal place. Names of variables were also shortened to 7 characters or less, as required for analysis. RDA was performed using Canoco v4.5 statistical software (ter Braak and Smilauer, 2002).

5.3 Results

5.3.1 Rothamsted Collection Data and Acquired Climate Data

The Rothamsted trap was active during the summer seasons of 2013 to 2017. All Ceratopogonidae collection data, organized by week as detailed in Materials and Methods, are reported in Tables 5.1 to 5.5. Weather data were acquired from Environment and Climate Change Canada (weather.gc.ca) and used for general trend analysis as well as for RDA generation is supplied in Tables 5.6 to 5.8.

Table 5.1. Ceratopogonidae captured by the Rothamsted Trap during the summer season of 2013, organized by genus. Data are compounded on a weekly basis to account for lack of separate daily samples on weekends. Collection began on epiweek 18.5 and ended on epiweek 41.5.

	May 1-7	May 8-14	May 15-21	May 22-28	May 29-Jun 4	Jun 5-Jun 11	Jun 12-Jun 18	Jun 19-Jun 25	Jun 26-Jul 2	Jul 3-Jul 9	Jul 10-Jul 16	Jul 17-23
Alluaudomyia	0	0	0	0	0	0	0	3	1	2	3	3
Atrichopogon	0	0	86	42	61	8	5	4	8	143	83	25
Bezzia	0	0	0	0	12	5	33	139	116	333	176	79
Culicoides	5	14	67	21	19	18	34	167	521	190	64	16
Dasyhelea	2	7	20	16	4	11	4	20	25	38	30	17
Forcipomyia	0	0	0	1	0	4	4	26	10	39	56	19
Johannsenomyia	0	0	0	0	0	0	0	0	0	0	0	4
Mallochohelea	0	0	1	0	7	7	7	2	0	17	11	3
Nilobezzia	0	0	0	0	2	2	4	2	5	54	19	10
Palpomyia	0	0	0	0	0	0	0	0	0	0	0	0
Phaenobezzia	0	0	0	0	0	1	10	20	83	62	24	6
Probezzia	0	0	0	0	0	0	15	68	34	138	8	0
Sphaeromias	0	0	0	0	0	0	0	0	0	0	0	0
Stilobezzia	0	0	0	0	0	0	0	0	0	1	2	1

	Jul 24-30	Jul 31-Aug 6	Aug 7-13	Aug 14-20	Aug 21-27	Aug 28-Sept 3	Sept 4-10	Sept 11-17	Sept 18-24	Sept 25-Oct1	Oct 2-8
Alluaudomyia	4	0	3	3	1	1	1	0	0	0	0
Atrichopogon	12	14	24	23	15	17	4	12	1	1	6
Bezzia	63	48	80	95	184	187	18	2	0	0	0
Culicoides	17	8	16	6	7	1	2	2	0	1	1
Dasyhelea	35	12	22	9	43	9	7	7	1	6	13
Forcipomyia	36	37	26	68	70	19	45	32	40	9	40
Johannsenomyia	0	0	0	0	0	0	0	0	0	0	0
Mallochohelea	0	1	1	0	1	0	0	0	0	0	0
Nilobezzia	11	4	4	9	1	0	0	0	0	0	0
Palpomyia	0	0	1	1	3	0	0	0	0	0	0
Phaenobezzia	3	4	11	26	17	0	1	0	0	0	0
Probezzia	2	0	1	0	0	0	0	0	0	0	0
Sphaeromias	0	0	2	2	0	0	0	0	0	0	0
Stilobezzia	0	0	0	0	0	0	0	0	0	0	0

Table 5.2. Ceratopogonidae captured by the Rothamsted Trap during the summer season of 2014, organized by genus. Data are compounded on a weekly basis to account for lack of separate daily samples on weekends. Collection began on epiweek 20.5 and ended on epiweek 41.5.

	May 14-20	May 21-27	May 28-Jun 3	Jun 4-10	Jun 11-17	Jun 18-24	Jun 25-Jul 1	Jul 2-8	Jul 9-15	Jul 16-22	Jul 23-29
Alluaudomyia	0	0	6	3	1	9	4	2	1	22	13
Atrichopogon	2	2	106	53	43	41	86	195	53	61	42
Bezzia	0	1	13	7	21	29	123	106	399	921	450
Ceratopogon	0	0	0	1	0	0	0	0	0	0	0
Culicoides	3	18	317	480	798	348	357	134	57	16	6
Dasyhelea	34	47	112	84	53	70	130	58	38	67	27
Forcipomyia	0	2	6	5	32	26	38	44	43	79	49
Jenkinshelea	0	0	0	0	0	0	0	0	0	0	1
Mallochohelea	0	0	0	1	5	11	3	2	1	0	0
Nilobezzia	0	0	0	0	0	0	16	6	18	61	36
Palpomyia	0	0	0	6	1	0	1	1	0	5	5
Parabezzia	0	0	0	4	0	0	1	1	0	0	0
Phaenobezzia	0	0	1	0	1	1	28	30	70	58	7
Probezzia	0	0	0	0	1	37	88	16	15	25	7
Sphaeromias	0	0	0	0	0	0	15	6	1	11	3
Stilobezzia	0	0	0	0	0	0	1	1	1	0	0

	Jul 30-Aug 5	Aug 6-12	Aug 13-19	Aug 20-26	Aug 27-Sept 2	Sept 3-9	Sep 10-16	Sep 17-23	Sep 24-30	Oct 1-7
Alluaudomyia	27	6	0	2	3	1	0	0	0	0
Atrichopogon	18	27	24	23	14	10	1	0	1	0
Bezzia	261	111	8	108	87	35	1	0	0	0
Ceratopogon	0	0	0	0	0	0	0	0	0	0
Culicoides	3	7	7	7	7	1	1	0	0	0
Dasyhelea	27	77	38	37	15	20	8	8	7	1
Forcipomyia	34	27	31	39	51	18	16	12	20	6
Jenkinshelea	0	0	0	0	1	0	0	0	0	0
Mallochohelea	0	25	3	0	0	0	0	0	0	0
Nilobezzia	16	12	4	2	0	0	0	0	0	0
Palpomyia	10	5	5	1	0	1	0	0	0	0
Parabezzia	0	0	0	0	0	0	0	0	0	0
Phaenobezzia	6	6	4	17	4	0	0	0	0	0
Probezzia	5	0	0	0	0	0	0	0	0	0
Sphaeromias	3	0	0	0	0	0	0	0	0	0
Stilobezzia	1	0	0	0	0	0	0	0	0	0

Table 5.3. Ceratopogonidae captured by the Rothamsted Trap during the summer season of 2015, organized by genus. Data are compounded on a weekly basis to account for lack of separate daily samples on weekends. Collection began on epiweek 18.5 and ended on epiweek 40.5.

	May 6-12	May 13-19	May 20-26	May 27 - June 2	June 3-9	June 10-16	June 17-23	June 24-30	July 1-7	July 8-14	July 15-21
Alluaudomyia	0	1	2	2	2	2	1	3	2	0	0
Atrichopogon	0	5	2	2	1	4	3	7	7	9	17
Bezzia	0	1	2	4	10	23	59	94	99	211	329
Ceratopogon	0	1	0	0	0	0	0	0	0	0	0
Clinohelea	0	0	0	0	0	0	0	0	0	0	0
Culicoides	7	13	3	14	15	20	76	823	417	148	32
Dasyhelea	20	28	8	10	1	7	18	25	15	17	10
Echinohelea	0	0	0	0	0	0	0	0	3	0	0
Forcipomyia	12	1	0	2	1	3	5	9	18	13	17
Jenkinshelea	0	0	0	0	0	0	0	0	0	0	1
Johannsenomyia	0	0	0	0	0	0	0	0	0	0	0
Mallochohelea	0	0	0	0	0	40	51	13	7	0	0
Nilobezzia	0	0	0	0	0	0	6	5	7	22	74
Palpomyia	0	0	0	0	0	1	1	0	2	0	1
Phaenobezzia	0	0	0	0	0	0	12	31	19	38	13
Probezzia	0	0	0	0	0	121	822	479	341	50	25
Sphaeromias	0	0	0	0	0	1	2	1	7	2	5
Stilobezzia	0	0	0	0	1	0	0	0	0	2	3
	July 22-28	**July 29-Aug 4**	**Aug 5-11**	**Aug 12-18**	**Aug 19-25**	**Aug 26-Sept 1**	**Sept 2-8**	**Sept 9-15**	**Sept 16-22**	**Sept 23-29**	**Sept 30-Oct 6**
Alluaudomyia	0	3	0	3	0	3	2	0	0	0	0
Atrichopogon	17	6	4	4	18	14	8	6	4	3	1
Bezzia	144	51	6	18	31	67	20	0	1	0	0
Ceratopogon	0	0	0	0	0	0	0	0	0	0	0
Clinohelea	1	0	0	0	0	0	0	0	0	0	0
Culicoides	27	10	1	0	2	2	3	0	2	2	0
Dasyhelea	15	14	3	5	5	21	23	5	4	7	1
Echinohelea	0	0	0	0	0	0	0	0	0	0	0
Forcipomyia	24	17	7	34	38	99	41	5	14	9	5
Jenkinshelea	2	0	0	4	1	0	0	0	0	0	0
Johannsenomyia	3	1	0	0	0	0	0	0	0	0	0
Mallochohelea	0	6	0	3	0	0	0	0	0	0	0
Nilobezzia	82	37	15	21	5	1	1	0	0	0	0
Palpomyia	6	1	6	2	5	1	2	0	0	0	0
Phaenobezzia	4	3	0	5	10	3	1	0	0	0	0
Probezzia	8	0	0	0	0	0	0	0	0	0	0
Sphaeromias	7	4	1	1	2	0	0	0	0	0	0
Stilobezzia	0	2	1	0	0	1	0	0	0	0	0

Table 5.4. Ceratopogonidae captured by the Rothamsted Trap during the summer season of 2016, organized by genus. Data are compounded on a weekly basis to account for lack of separate daily samples on weekends. Collection began on epiweek 21.5 and ended on epiweek 40.5.

	May 25-31	June 1-7	June 8-14	June 15-21	June 22-28	June 29-July 5	July 6-12	July 13-19	July 20-26	July 27-Aug 2
Alluaudomyia	5	0	0	3	0	7	7	6	2	0
Atrichopogon	3	3	3	1	1	4	10	3	3	1
Bezzia	22	21	15	53	24	99	128	34	17	1
Culicoides	239	96	21	82	30	68	25	11	8	5
Dasyhelea	15	6	2	9	4	12	15	10	13	6
Forcipomyia	7	2	0	8	7	8	12	16	15	6
Jenkinshelea	0	0	0	0	0	1	0	2	0	0
Johannsenomyia	0	0	0	0	0	0	1	9	1	0
Mallochohelea	0	1	8	23	0	1	1	2	1	0
Monohelea	0	0	0	0	0	1	0	0	0	0
Nilobezzia	0	0	0	1	0	25	35	35	14	14
Palpomyia	0	0	0	0	0	1	5	7	4	5
Phaenobezzia	0	0	0	2	15	32	8	4	0	1
Probezzia	0	0	6	307	103	193	63	3	1	0
Sphaeromias	0	0	0	2	42	119	53	28	6	1
Stilobezzia	0	1	0	0	2	3	12	2	0	0

	Aug 3-9	Aug 10-16	Aug 17-23	Aug 24-30	Aug 31-Sept 6	Sept 7-13	Sept 14-20	Sept 21-27	Sept 28-Oct 4
Alluaudomyia	1	0	2	3	0	2	0	0	0
Atrichopogon	6	5	2	2	2	2	0	0	1
Bezzia	3	7	15	27	6	4	1	0	0
Culicoides	0	3	5	4	4	5	4	2	3
Dasyhelea	24	21	3	4	6	14	5	4	7
Forcipomyia	9	7	13	9	17	16	39	18	5
Jenkinshelea	1	3	0	0	0	1	0	0	0
Johannsenomyia	2	0	0	0	0	0	0	0	0
Mallochohelea	0	0	0	0	0	0	0	0	0
Monohelea	0	0	0	0	0	0	0	0	0
Nilobezzia	16	2	1	4	0	0	0	0	0
Palpomyia	3	1	0	0	0	0	0	0	0
Phaenobezzia	1	23	12	2	0	0	0	0	0
Probezzia	0	0	0	0	0	0	0	0	0
Sphaeromias	3	0	0	0	0	0	0	0	0
Stilobezzia	0	0	0	0	0	1	0	0	0

Table 5.5. Ceratopogonidae captured by the Rothamsted Trap during the summer season of 2017, organized by genus. Data are compounded on a weekly basis to account for lack of separate daily samples on weekends. Collection began on epiweek 18.5 and ended on epiweek 40.5.

	May 3-9	May 10-16	May 17-23	May 24-30	May 31-June 6	June 7-13	June 14-20	June 21-27	June 28-July 4	July 5-11	July 12-18
Alluaudomyia	0	0	0	0	0	0	1	0	0	1	3
Atrichopogon	0	0	3	0	0	5	2	5	7	5	3
Bezzia	0	1	1	5	5	19	43	24	70	112	259
Ceratopogon	0	0	0	0	0	0	0	0	0	1	0
Culicoides	0	5	14	52	38	44	483	64	113	12	14
Dasyhelea	0	20	6	27	6	5	8	7	15	21	15
Forcipomyia	0	1	0	2	1	9	14	30	47	48	43
Jenkinshelea	0	0	0	0	0	0	0	0	1	3	0
Johannsenomyia	0	0	0	0	0	0	0	0	0	2	3
Mallochohelea	0	0	0	0	2	7	3	1	0	0	1
Nilobezzia	0	0	0	0	0	0	0	0	20	79	117
Palpomyia	0	0	0	0	0	1	4	0	0	2	9
Phaenobezzia	0	0	0	0	0	0	27	28	75	80	60
Probezzia	0	0	0	0	1	2	64	42	202	37	6
Sphaeromias	0	0	0	0	0	0	2	7	40	34	21
Stilobezzia	0	0	0	0	0	0	0	0	0	1	2
	July 19-25	**July 26-Aug 1**	**Aug 2-8**	**Aug 9-15**	**Aug 16-22**	**Aug 23-29**	**Aug 30-Sept 5**	**Sept 6-12**	**Sept 13-19**	**Sept 20-26**	**Sept 27-Oct 3**
Alluaudomyia	0	1	0	0	1	0	4	0	0	0	0
Atrichopogon	7	6	10	18	16	17	17	16	9	20	13
Bezzia	135	68	66	109	114	17	38	26	7	2	0
Ceratopogon	0	0	0	0	0	0	0	0	0	0	0
Culicoides	6	9	8	10	8	6	7	4	4	4	0
Dasyhelea	19	29	41	65	42	48	39	25	47	30	27
Forcipomyia	32	36	21	27	18	23	24	21	46	44	11
Jenkinshelea	4	1	2	5	3	0	0	0	0	1	0
Johannsenomyia	0	0	1	1	0	0	0	0	0	0	0
Mallochohelea	2	2	3	0	0	0	0	0	0	0	0
Nilobezzia	49	26	19	13	2	0	0	0	0	0	0
Palpomyia	2	13	13	9	2	0	0	0	0	0	0
Phaenobezzia	16	24	7	65	19	8	6	2	3	0	0
Probezzia	5	1	0	0	0	0	0	0	0	0	0
Sphaeromias	8	3	4	1	0	0	0	0	0	0	0
Stilobezzia	3	2	0	0	0	0	0	0	0	0	0

Table 5.6. Weather data per week of compounded collection data for 2013 and 2014. Variables include weekly averages of temperature, relative humidity, wind speed, air pressure, and precipitation.

2013	Temp (˚C)	Rel Hum (%)	Wind Spd (km/h)	Pressure (kPa)	Precip (mm)
May 1-7	15.0	50.2	13.3	101.0	0.0
May 8-14	12.0	68.6	19.0	100.1	0.6
May 15-21	17.8	56.9	17.2	100.2	0.5
May 22-28	14.0	62.1	17.2	100.6	4.9
May 29-Jun 4	20.8	65.8	21.5	100.3	6.3
Jun 5-Jun 11	16.2	81.4	12.6	100.2	6.0
Jun 12-Jun 18	19.1	70.9	15.4	100.0	3.0
Jun 19-Jun 25	22.5	61.2	16.5	100.7	0.3
Jun 26-Jul 2	20.9	80.1	12.9	99.7	5.1
Jul 3-Jul 9	24.3	79.2	14.1	100.6	2.7
Jul 10-Jul 16	24.4	71.1	11.7	100.8	0.7
Jul 17-23	25.5	68.5	16.6	100.1	7.8
Jul 24-30	19.3	65.1	13.4	100.5	0.3
Jul 31-Aug 6	20.3	65.0	14.3	100.3	2.5
Aug 7-13	20.6	68.7	14.5	100.3	4.7
Aug 14-20	19.8	65.0	12.1	100.8	0.0
Aug 21-27	22.1	69.3	14.4	100.7	3.2
Aug 28-Sept 3	22.6	80.3	13.8	99.9	0.1
Sept 4-10	18.2	66.9	17.0	100.6	1.4
Sept 11-17	15.5	72.8	16.1	100.7	0.8
Sept 18-24	15.0	71.8	12.8	100.4	6.0
Sept 25-Oct 1	15.9	76.0	12.5	100.7	0.0
Oct 2-8	16.8	78.1	12.1	100.4	4.0
2014	Temp (˚C)	Rel Hum (%)	Wind Spd (km/h)	Pressure (kPa)	Precip (mm)
May 14-20	13.1	72.7	14.2	100.6	1.3
May 21-27	17.9	67.7	15.4	100.3	2.0
May 28-Jun 3	18.6	65.2	16.8	100.7	1.9
Jun 4-10	18.1	64.2	11.7	100.2	0.5
Jun 11-17	20.1	75.3	15.2	100.2	5.6
Jun 18-24	20.6	65.4	11.6	100.4	0.3
Jun 25-Jul 1	24.4	71.4	16.7	100.3	2.3
Jul 2-8	21.7	67.7	19.8	100.1	6.0
Jul 9-15	20.7	67.1	13.7	100.2	1.4
Jul 16-22	20.4	70.7	11.0	100.7	1.0
Jul 23-29	20.6	65.7	15.6	100.0	18.5
Jul 30-Aug 5	21.0	76.1	11.0	100.6	1.0
Aug 6-12	20.7	67.7	12.4	100.5	0.5
Aug 13-19	18.0	67.8	17.1	100.0	0.0
Aug 20-26	21.9	75.2	14.7	100.5	0.0
Aug 27-Sept 2	22.4	69.1	17.2	100.4	0.2
Sept 3-9	20.6	68.4	14.1	100.6	3.4
Sep 10-16	14.5	72.7	13.8	100.7	1.8
Sep 17-23	14.9	68.2	18.2	100.5	0.5
Sep 24-30	16.4	77.1	7.5	101.0	0.0
Oct 1-7	14.8	77.9	19.6	99.7	2.4

Table 5.7. Weather data per week of compounded collection data for 2015 and 2016. Variables include weekly averages of temperature, relative humidity, wind speed, air pressure, and precipitation.

2015	Temp (˚C)	Rel Hum (%)	Wind Spd (km/h)	Pressure (kPa)	Precip (mm)
May 6-12	17.2	70.3	12.4	100.6	1.9
May 13-19	14.7	68.9	13.2	100.8	0.0
May 20-26	15.1	53.6	19.2	100.8	0.0
May 27 - June 2	17.1	76.7	17.6	100.8	6.3
June 3-9	16.5	76.8	13.1	100.2	4.1
June 10-16	19.1	84.3	11.6	100.1	3.1
June 17-23	19.4	75.9	13.9	100.2	0.0
June 24-30	17.7	79.5	13.0	100.2	7.8
July 1-7	19.5	74.4	10.8	100.3	0.2
July 8-14	20.0	77.4	9.6	100.2	2.0
July 15-21	21.1	75.2	14.6	99.9	0.7
July 22-28	22.2	64.5	11.7	100.2	0.0
July 29-Aug 4	22.9	69.4	15.2	99.8	1.2
Aug 5-11	18.9	77.2	10.8	100.3	1.3
Aug 12-18	22.9	78.1	14.0	100.5	0.5
Aug 19-25	20.4	73.0	13.4	100.2	1.3
Aug 26-Sept 1	20.0	79.1	9.3	100.7	0.0
Sept 2-8	25.2	78.1	11.9	100.4	5.5
Sept 9-15	17.3	77.0	15.4	100.2	6.6
Sept 16-22	18.4	76.8	12.3	100.7	0.0
Sept 23-29	17.8	83.3	11.7	101.2	2.7
Sept 30-Oct 6	11.6	76.7	17.6	101.2	0.3
2016	Temp (˚C)	Rel Hum (%)	Wind Spd (km/h)	Pressure (kPa)	Precip (mm)
May 25-31	22.7	64.9	15.2	100.4	0.0
June 1-7	18.7	71.4	16.3	99.8	2.0
June 8-14	16.5	55.9	16.2	100.2	0.0
June 15-21	21.5	54.8	13.9	100.3	0.7
June 22-28	21.5	59.8	13.7	100.3	0.0
June 29-July 5	20.7	57.0	14.3	100.3	0.0
July 6-12	24.2	70.1	12.4	100.0	0.0
July 13-19	23.3	61.5	17.7	100.3	0.0
July 20-26	25.3	61.4	14.2	100.3	4.2
July 27-Aug 2	23.3	74.2	11.3	100.4	2.7
Aug 3-9	24.3	63.8	12.0	100.3	0.2
Aug 10-16	26.7	76.8	15.6	100.3	3.4
Aug 17-23	23.0	68.2	13.5	100.3	0.8
Aug 24-30	23.8	71.8	12.0	100.7	1.5
Aug 31-Sept 6	20.0	72.2	10.3	100.8	0.5
Sept 7-13	23.3	74.3	13.9	100.3	2.7
Sept 14-20	19.1	78.0	11.9	100.7	1.5
Sept 21-27	16.8	70.1	12.3	100.7	1.3
Sept 28-Oct 4	16	81.7	13.6	100.8	4.2

Table 5.8. Weather data per week of compounded collection data for 2017. Variables include weekly averages of temperature, relative humidity, wind speed, air pressure, and precipitation.

2017	Temp (˚C)	Rel Hum (%)	Wind Spd (km/h)	Pressure (kPa)	Precip (mm)
May 3-9	7.7	75.2	16.7	99.8	8.4
May 10-16	11.8	72.0	12.2	100.2	0.0
May 17-23	16.5	67.5	21.3	100.3	2.4
May 24-30	16.8	81.0	11.8	99.6	7.1
May 31-June 6	15.5	71.2	13.2	100.1	0.7
June 7-13	20.4	64.2	16.9	100.2	0.0
June 14-20	22.0	69.7	15.9	99.8	1.2
June 21-27	18.6	74.6	15.0	100.0	11.4
June 28-July 4	21.5	69.8	15.4	100.3	1.3
July 5-11	21.3	72.6	12.7	100.1	0.7
July 12-18	21.6	83.6	11.5	100.3	4.1
July 19-25	21.5	80.1	10.7	100.1	3.8
July 26-Aug 1	21.2	76.5	9.8	100.5	2.4
Aug 2-8	20.6	79.6	14.4	100.3	4.5
Aug 9-15	21.2	77.5	11.1	100.4	2.0
Aug 16-22	22.0	78.0	15.7	100.1	0.0
Aug 23-29	17.2	69.3	10.6	100.8	3.7
Aug 30-Sept 5	16.5	74.3	14.5	100.3	4.7
Sept 6-12	14.4	76.3	9.4	100.9	0.1
Sept 13-19	20.1	83.0	7.8	100.5	0.0
Sept 20-26	22.8	78.4	7.9	100.6	0.0
Sept 27-Oct 3	15.2	70.2	14.9	101.1	0.1

5.3.2 Genera Displaying the Most Prominent Overall Abundance

Throughout the entirety of the Rothamsted trap's time of operation during the summer months of 2013 to 2017, the three Ceratopogonidae genera displaying highest abundance were *Bezzia* (n = 7019), *Culicoides* (n = 6893), and *Probezzia* (n = 3342; Tables 5.1 – 5.5). These genera are the primary focus of analysis due to their prominence in the trap samples.

5.3.3 Yearly Trends in *Bezzia, Culicoides,* and *Probezzia*

5.3.3.1 2013

A large peak in *Culicoides* abundance was recorded in June to July of this year, with a notable increase beginning during the 7-day period of June 12th to June 18th (epiweek 24.5), and the population dropping to low numbers between July 17th and July 23rd (epiweek 29.5). During the week at which the absolute peak of this incident occurred, 521 *Culicoides* specimens were captured. The genus *Bezzia* also exhibited a population boom, peaking during the week of July 3rd to July 9th (epiweek 27.5), with a secondary population spike occurring between August 21st and September 3rd (epiweek 35.5). In the case of *Probezzia*, only a relatively small population spike occurred during July 3rd to July 9th (epiweek 27.5). These trends can be observed in Figure 5.2.

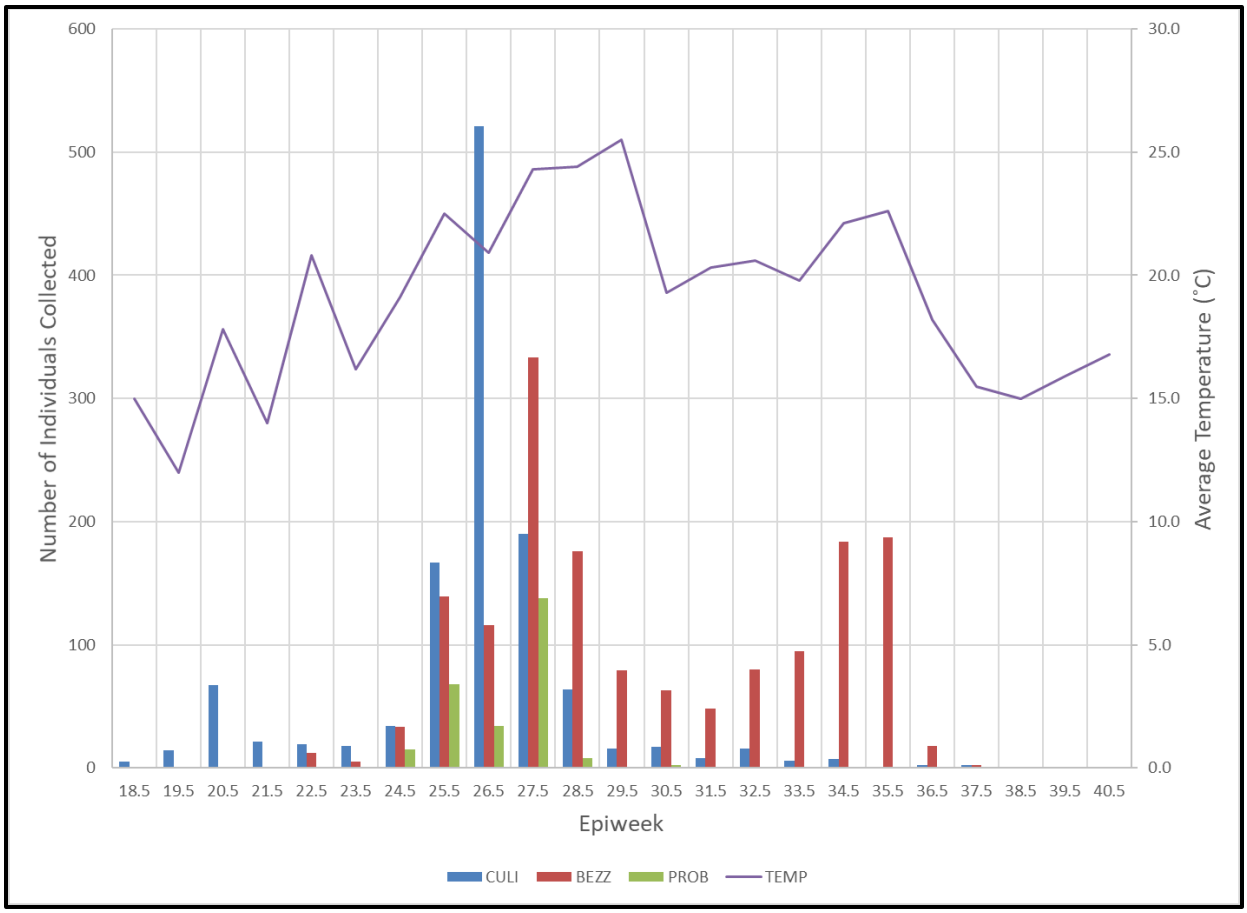

Figure 5.2. Average temperature (TEMP) and abundance of three biting midge genera (*Bezzia*, BEZZ; *Culicoides*, CULI; and *Probezzia*, PROB) per epiweek during Rothamsted trap operation of 2013.

5.3.3.2 2014

In 2014, the peak amount of *Culicoides* captured was recorded between June 11th and 17th (epiweek 24.5), with an increase in population beginning two weeks prior (May 28th to June 3rd; epiweek 22.5), and the peak diminishing roughly 4 weeks afterward (week of July 9th to 15th; epiweek 28.5). A population spike for *Bezzia* occurred, with the peak value reaching even higher than that observed in *Culicoides* (921 individuals, compared to the maximum of 798 observed in *Culicoides*). This spike began between June 11th and 17th (epiweek 24.5) and numbers tapered off by August 19th (epiweek 33.5). A relatively small peak in *Probezzia* abundance was observed,

but only reached a maximum of 88 individuals during the week of June 25 to July 1 (epiweek 26.5).

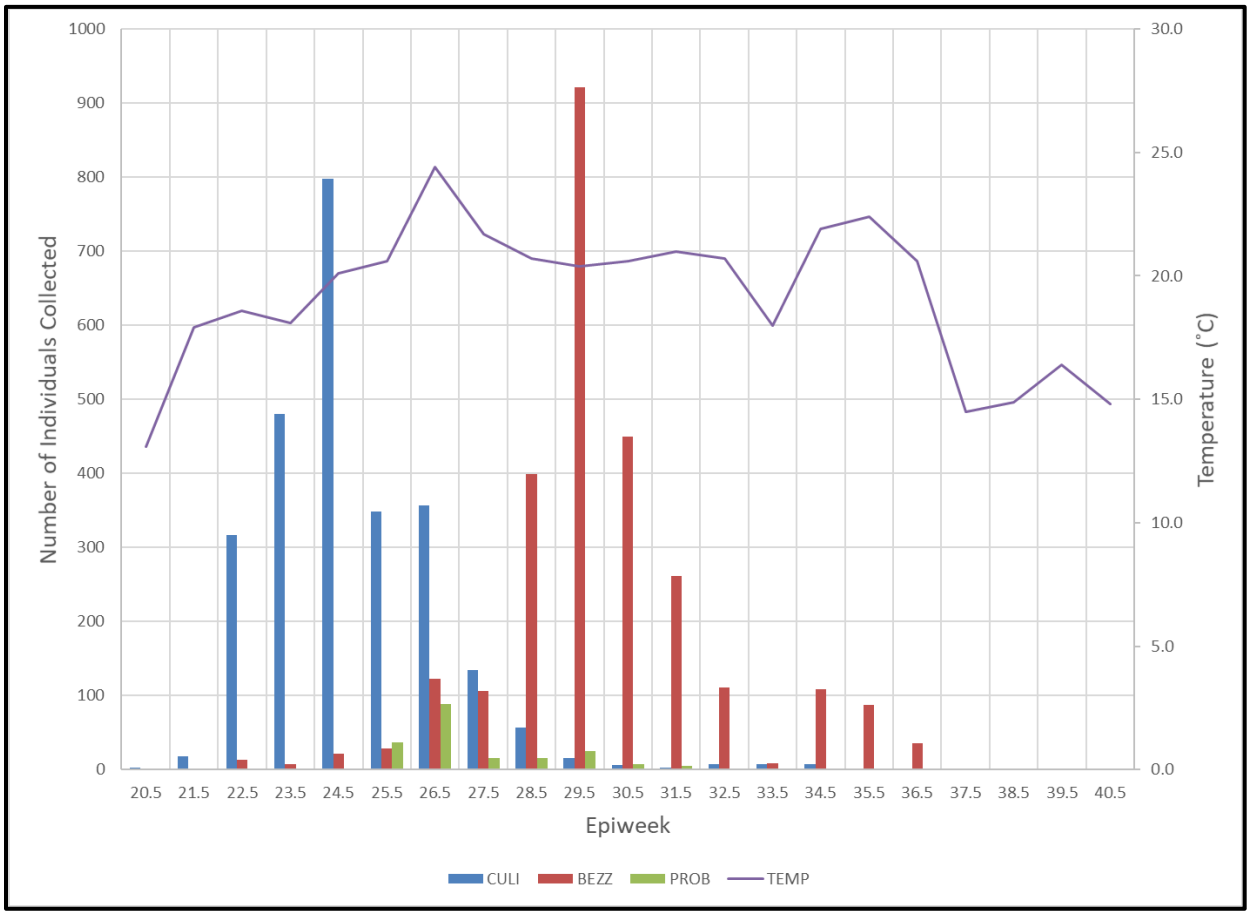

Figure 5.3. Average temperature (TEMP) and abundance of three biting midge genera (*Bezzia*, BEZZ; *Culicoides*, CULI; and *Probezzia*, PROB) per epiweek during Rothamsted trap operation of 2014.

5.3.3.3 2015

A *Culicoides* population spike was once again observed in 2015, with an increase in collected specimens beginning as early as the week of June 17th to 23rd (epiweek 24.5). The peak abundance of 823 collected individuals was reached by June 24th to 30th (epiweek 25.5), and the population diminished again by July 15th to 21st (epiweek 28.5), constituting a month-long event in which there were high numbers of *Culicoides*. *Bezzia*'s yearly population spike was observed

during July 15th to 21st (epiweek 28.5) with 329 individuals collected, with a smaller second population spike during August 26th to September 1st (epiweek 34.5). This year saw the highest recorded population spike of *Probezzia*, where the maximum of 822 collected individuals occurred one week before the *Culicoides* peak was observed (June 17th to 23rd; epiweek 24.5). Additionally, the observed maximum population reached by *Probezzia* was approximately the same number of individuals as was observed in *Culicoides* (n = 823).

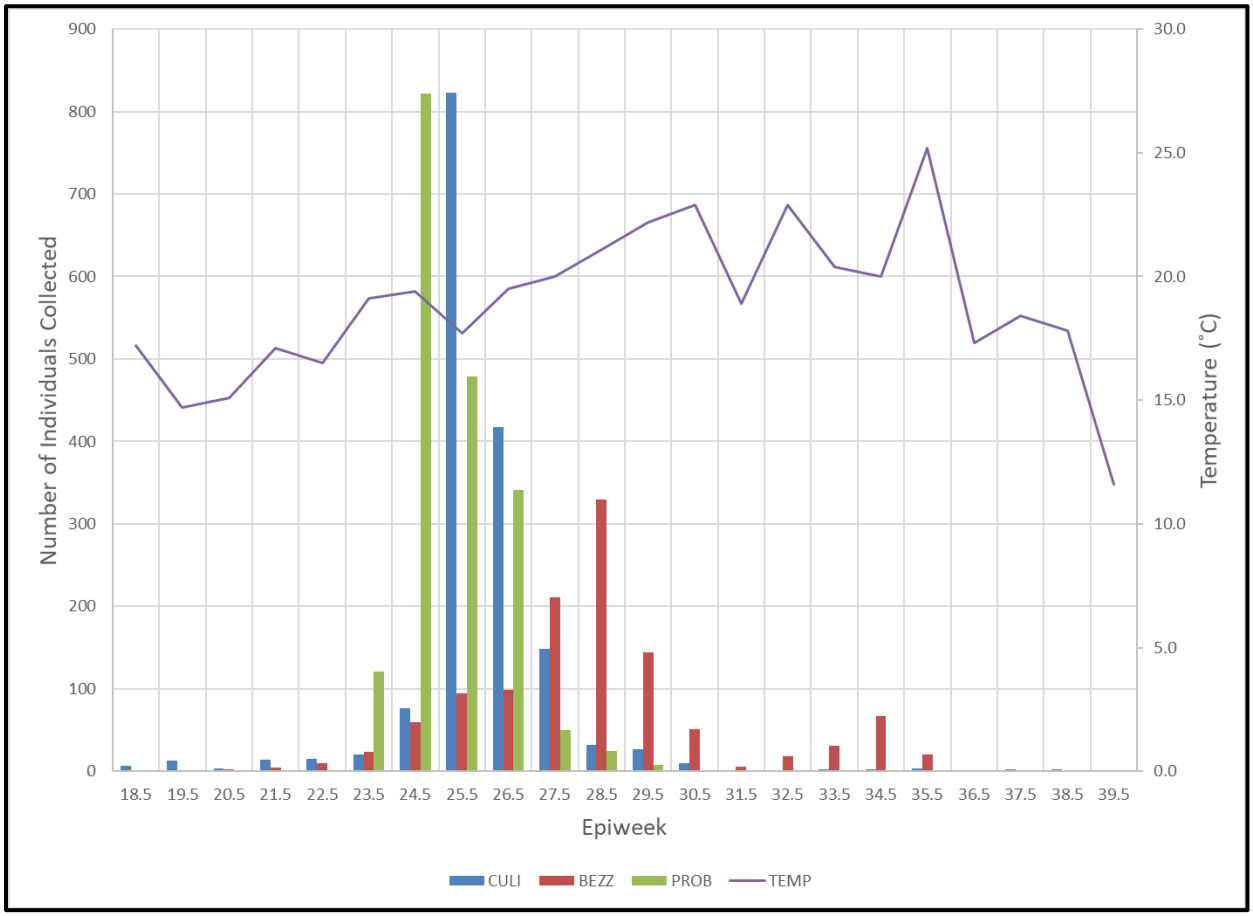

Figure 5.4. Average temperature (TEMP) and abundance of three biting midge genera (*Bezzia*, BEZZ; *Culicoides*, CULI; and *Probezzia*, PROB) per epiweek during Rothamsted trap operation of 2015.

5.3.3.4 2016

One important note regarding the 2016 Rothamsted data is that collection did not begin until approximately the last week of May, with data beginning on May 25th (epiweek 21.5). Observed trends from 2016 data show the highest number of *Culicoides* were collected in the very first week of Rothamsted activation (epiweek 21.5), with a peak value of 239, significantly lower than the peak values of every other year (2013 = 521; 2014 = 798; 2015 = 823; 2017 = 483). *Bezzia* saw its primary population spike of 128 individuals during the week of July 6th to 12th (epiweek 27.5), followed once again by a much smaller spike in late August (Aug 24th to 30th; epiweek 34.5). *Probezzia* peak abundance was recorded from June 15th to 21st (epiweek 24.5).

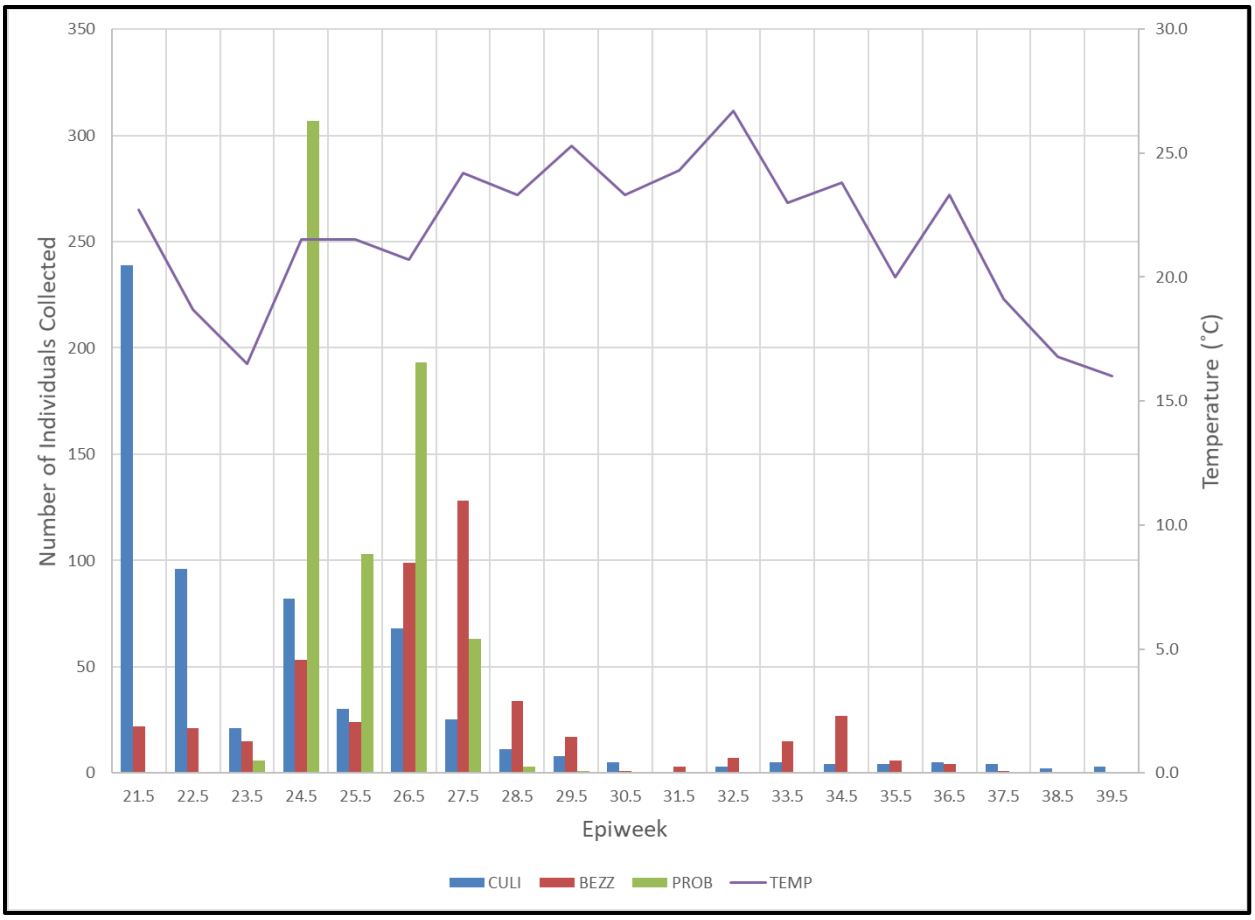

Figure 5.5. Average temperature (TEMP) and abundance of three biting midge genera (*Bezzia*, BEZZ; *Culicoides*, CULI; and *Probezzia*, *PROB*) per epiweek during Rothamsted trap operation of 2016.

5.3.3.5 2017

In the final sampling season, *Culicoides*' major population spike was observed June 13th to 20th (epiweek 24.5). *Bezzia* trends produced a maximum abundance during July 12th to 18th (epiweek 28.5), followed by a smaller secondary peak occurring August 16th to 22nd (epiweek 33.5). *Probezzia*'s yearly spike was observed in between population spikes of *Culicoides* and *Bezzia*, occurring from June 28th to July 4th (epiweek 26.5).

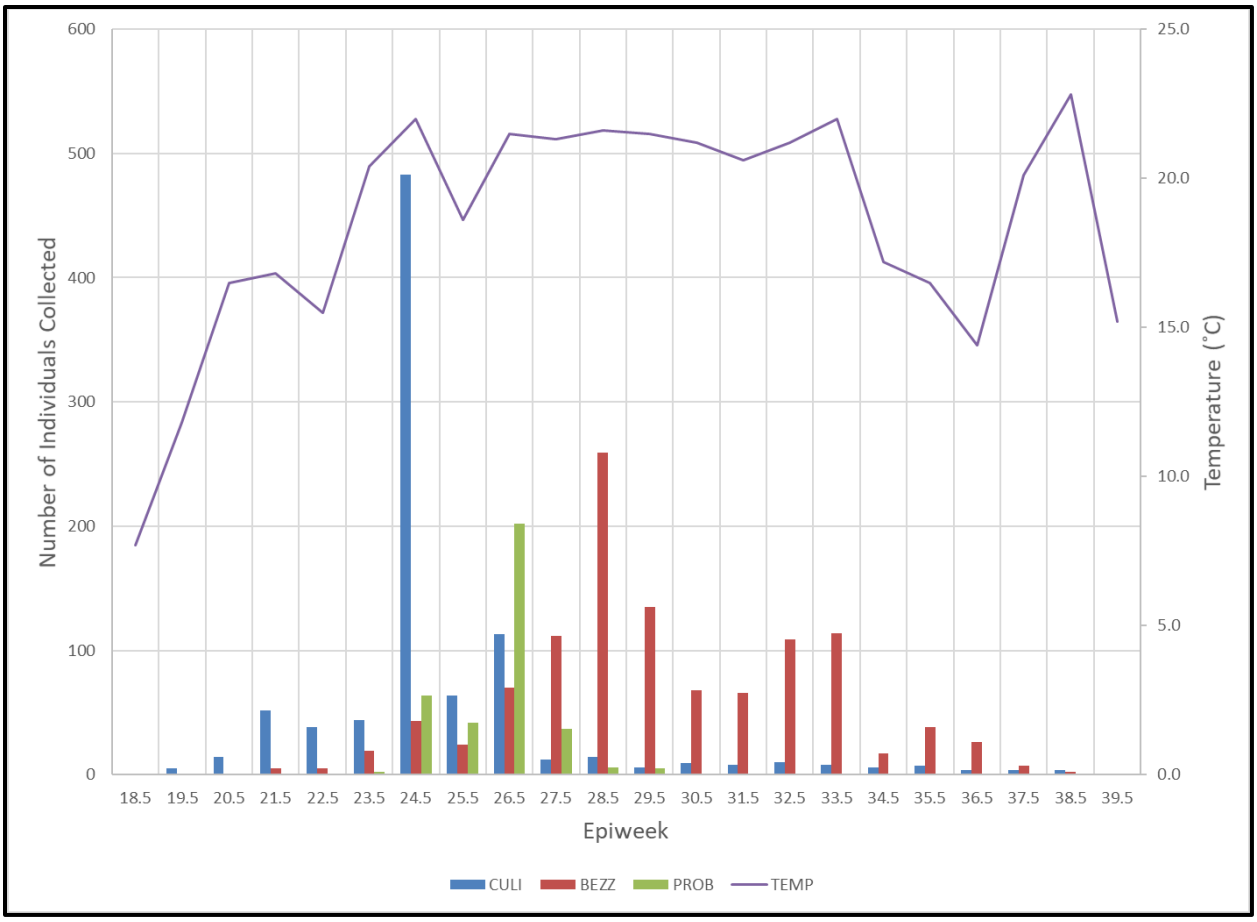

Figure 5.6. Average temperature (TEMP) and abundance of three biting midge genera (*Bezzia*, BEZZ; *Culicoides*, CULI; and *Probezzia*, PROB) per epiweek during Rothamsted trap operation of 2017.

5.3.4 RDA Analysis

RDA results are displayed in Figure 5.7. Climate and temporal variables are displayed as red arrow vectors, while various genera of Ceratopogonidae are displayed as dark blue arrow vectors. There are 107 data points, with each point representing 1 week of collection data. These data points are representative of the cumulative variance for all variables for each week of data and were used to create arrow vectors. Relatedness of variables is visualized by the angle between those variables. Angles of smaller degrees indicate higher relatedness, while angles of 90 degrees suggest that the variables are not related. If vector arrows are separated by 180

degrees, it indicates that those variables are negatively correlated. Arrow vector length is representative of that variable's impact on the overall pattern of variance. Eigenvalues for Axis 1 (x-axis) and Axis 2 (y-axis) of the produced chart are 0.2746 and 0.0842, respectively. Overall, these axes account for 92.63% of variance in the genus and climate data, and this result is significant (Pseudo-F value = 8.9; P-value = 0.00062).

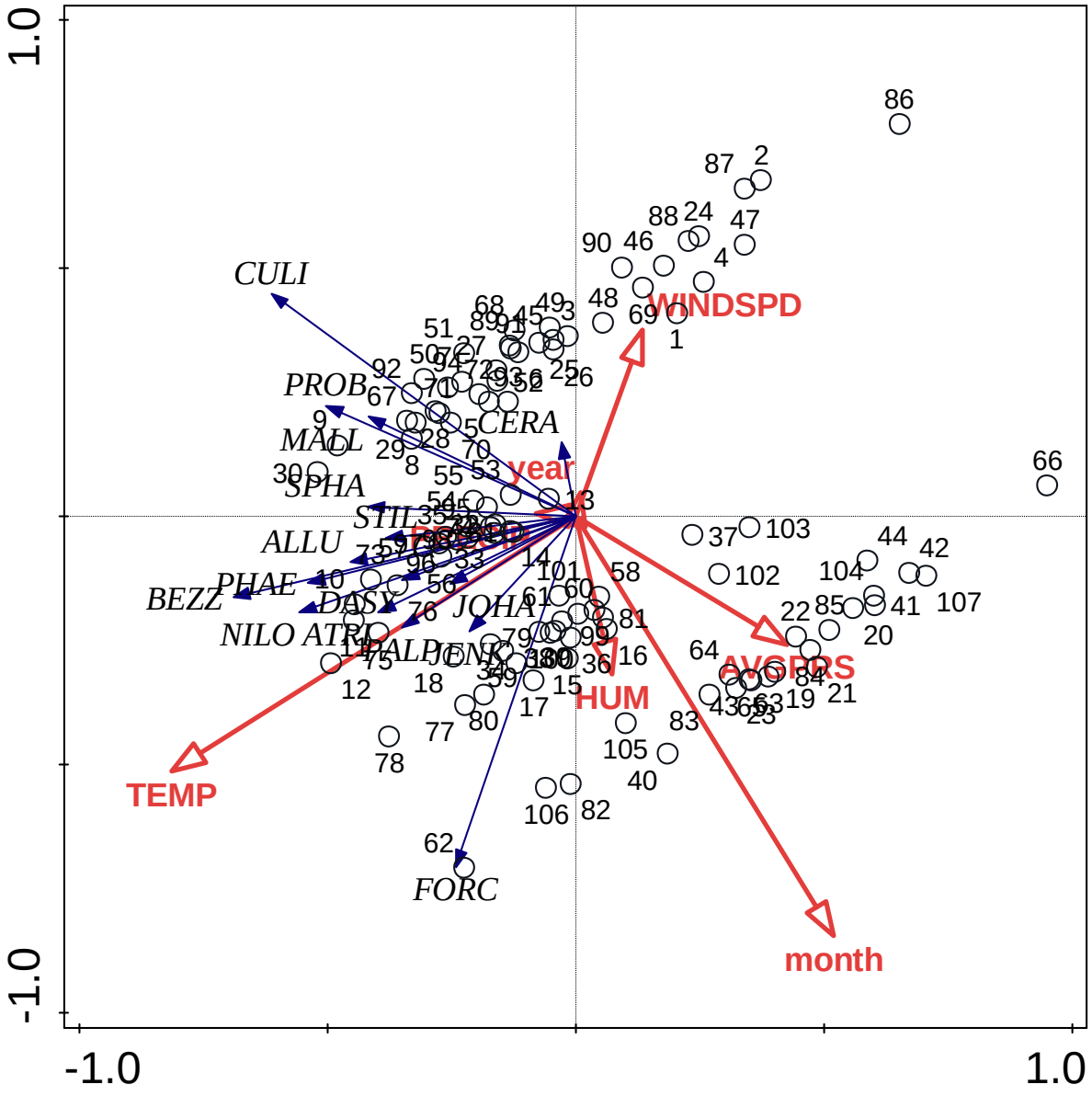

Figure 5.7. Redundancy analysis of weekly Rothamsted collection data from the summer seasons of 2013-2017. Data points 1 to 107 represent the variance of all variables calculated for each week of data. Red vector arrows display patterns of climate variables, with longer arrows representing stronger effects on observed variance (AVGPRS, Average Pressure [kPa]; HUM, Relative Humidity [%]; month, month of data collection; PRECIP, Precipitation [mm]; TEMP, Temperature [˚C]; WINDSPD, Wind Speed [km/h]; year, year of data collection). Dark blue arrow vectors represent Ceratopogonidae genera (ALLU, *Alluaudomyia*; ATRI, *Atrichopogon*; BEZZ, *Bezzia*; CERA, *Ceratopogon*; CULI; *Culicoides*; DASY, *Dasyhelea*; FORC, *Forcipomyia*; JENK, *Jenkinshelea*; JOHA, *Johannsenomyia*; MALL, *Mallochohelea*; NILO, *Nilobezzia*; PALP, *Palopmyia*; PHAE, *Phaenobezzia*; PROB, *Probezzia*; SPHA, *Sphaeromias*; STIL, *Stilobezzia*). Angle between two arrow vectors estimates the relatedness between those two variables. Arrows in the same direction (difference of 0 degrees) are completely positively correlated, while arrows in opposite directions (difference of 180 degrees) are completely negatively correlated. A difference of 90 degrees between two arrows indicates no correlation between represented variables.

5.4 Discussion

5.4.1 Yearly Trends in *Bezzia, Culicoides,* and *Probezzia*

5.4.1.1 2013

Interestingly, there were a number of days prior to the observed *Culicoides* population burst during which rainfall occurred. Precipitation was recorded for 9 of 15 days preceding the observed peak, which may have had an impact on *Culicoides* populations, as well as the overall pattern of this population increase. It is noted by Downes (1958) that North American *Culicoides* require semi-aquatic environments or moist areas in soil for egg deposition. This high amount of precipitation may have created a number of suitable environments for female *Culicoides* to lay eggs. Downes (1958) and Mellor *et al*. (2000) note that *Culicoides* may be able to complete larval development very quickly when exposed to a high ambient temperature, to the point where some *Culicoides* larvae progressed through all four of their larval stages and reached pupation in only four days.

Though southern Ontario is certainly not a tropical region, eggs laid by these ceratopogonids near the beginning of the time period with frequent precipitation (for example, around the week of May 29th to June 4th [epiweek 22.5], during which an average of 6.4mm of rain was recorded; Table 5.6) and a relatively high temperature may have still had enough time to develop completely into adult forms by the latter half of the *Culicoides* population spike. The temperature average during this population spike was reported as 20.9˚C, though the week beforehand displayed a higher average temperature of 22.5˚C; these values are relatively close to the maximum average temperature for this season, which was recorded as 25.5˚C. The higher average temperature recorded one week before the population spike may have facilitated rapid

development of additional *Culicoides*, which contributed to the genus' high abundance. However, it should also be noted that many *Culicoides* would have existed as larval forms at the start of this season, which is determined, presumably, by *Culicoides* activity in the previous year. As such, this population spike may only have been affected by temperature to a small degree, and instead was driven by a large number of first-generation insects undergoing a yearly pattern of mass emergence.

Bezzia population spikes observed in 2013 occurred during weeks exhibiting peaks in temperature, with the first spike occurring during an average temperature of 24.3˚C (only 1.2 degrees lower than the observed maximum) and the second spike occurring during a lower peak at approximately 22.1 and 22.6˚C. These trends indicate that there may be a direct association between temperature and the number of active *Bezzia*. Due to their recorded interactions with Chironomidae, it may also be that *Bezzia* are more active in times where chironomid activity is high; this may also co-ordinate with increased temperature. An examination of local chironomidae abundance during times of peak *Bezzia* populations may allow for assessment of this trend.

Probezzia peak populations coincided with the first observed spike in *Bezzia* abundance during a temperature of 24.3˚C. It is possible that this is directly related to the number of *Bezzia*, or potentially to other local insect populations displaying high activity or abundance in times of high temperature. Since *Probezzia* are predators of small insects (recorded as usually having a wing length up to a maximum of ~7mm; Wirth, 1994), including Chironomidae, they may display higher abundance in relation to prey. Additionally, it may be possible that *Bezzia* may function as prey for *Probezzia* and vice versa.

5.4.1.2 2014

The *Culicoides* peak recorded in 2014 correlates somewhat with an increase in precipitation (average precipitation = 5.6mm; Table 5.6), as observed in *Culicoides* during the 2013 season. This repeated observation may suggest somewhat of an association with local precipitation patterns.

After the primary population spike in observed *Bezzia* abundance, another small increase in population was seen shortly thereafter, with the population increasing again between August 20th and 26th (epiweek 34.5). Though the first *Bezzia* population spike does not occur during the same timeframe as a height in temperature, the second (though much smaller) spike appears to (average temperature = 21.9˚C). These trends somewhat align with those observed in 2013 *Bezzia* patterns.

Probezzia maximum abundance did not occur in conjunction with a spike in *Bezzia* populations, as was observed during 2013. No population spikes in this season appeared to occur simultaneously, as was observed with *Bezzia* and *Probezzia* in the previous year.

5.4.1.3 2015

Similar to the previous years, the week of the highest *Culicoides* abundance also displayed a relatively high amount of precipitation; in this case, an average of 7.8mm was recorded during the peak of this population spike, which was the highest recorded weekly precipitation average for the season (Table 5.7).

Bezzia once again exhibited a high abundance spike earlier in the season, followed by a smaller peak in abundance near late August. This particular event did not coincide directly with a high temperature as in previous years, though it was recorded just one week before the peak average temperature of the season. This may further support a relationship between temperature and *Bezzia* abundance.

Population spikes of both *Probezzia* and *Culicoides* do not appear to occur in relation to a high temperature in 2015, and are observed during average temperatures of lower than 20˚C (19.4˚C and 17.7˚C, respectively; max average temperature for the season was 25.2 during September 2 to 8 [epiweek 35.5]). It would be interesting to observe if co-occurrence between these two genera would reduce *Culicoides* numbers, as *Probezzia* are known to feed on small insects with a wing length as small as ~2mm (Wirth, 1994). High *Probezzia* abundance may also have directly influenced *Bezzia* populations by competition for similar food sources or by direct predation on *Bezzia* individuals, resulting in lower numbers of *Bezzia* by comparison. Downes (1978) noted that he had observed species of *Palpomyia* capturing and feeding upon *Bezzia* swarming within a group of Chironomidae, exemplifying possible cross-genus predation. He also notes that *Bezzia* and *Probezzia* species are both known to attack mayflies as well; therefore, it may be interesting to observe mayfly populations during timeframes when peak abundance of *Bezzia* and *Probezzia* occur.

5.4.1.4 2016

Data collection generally began near the beginning of May every year. Before the Rothamsted trap was activated for the summer season, a mechanic was required to conduct a

routine yearly inspection to ensure the trap was functioning properly and safely, at which point it could be turned on. In 2016, there were mechanical issues that needed to be repaired before the trap was activated, resulting in a late start to insect collection. Unfortunately, the delayed activation of the Rothamsted trap may have resulted in data loss, particularly with respect to the genus *Culicoides*. In every other year, there was a significant peak of captured *Culicoides* specimens at some point throughout the capture period. Generally, the peak number of *Culicoides* were collected in June to early July in all other years. This observation may indicate that the true peak of *Culicoides* abundance could have occurred during the time period before collection began, and was not sampled as a result of the Rothamsted trap being non-operational for maintenance.

Analysis of *Culicoides* trends with respect to weather variables, however, yields the observation that 2016's *Culicoides* population spike did not occur in conjunction with precipitation, with no precipitation recorded at all during that timeframe (0.0 mm; epiweek 21.5; Table 5.7). However, the average temperature during this spike event was relatively high at 22.7˚C; this is the first year where the initial temperature observation was over 20˚C. However, with the true timing of a peak in *Culicoides* abundance being uncertain, these relationships to weather variables may not be accurate.

Both population spikes of *Bezzia* appear to occur in correlation with relatively high average temperatures in the mid 20's (24.2˚C and 23.8˚C, respectively). The first population peak also occurred near the end of 2016's increase in *Probezzia*, which is similar to the trends observed in 2015. This may again indicate that *Probezzia* populations were suppressing *Bezzia* populations, possibly by outcompeting *Bezzia* specimens for resources, or by directly predating upon them. Once *Probezzia* became less abundant, it is possible that this allowed more *Bezzia* to

flourish. It should also be noted that of all initial *Bezzia* population spikes throughout the five sampling seasons, this particular spike had the lowest number of collected individuals.

5.4.1.5 2017

The observed abundance peak of *Culicoides* in 2017 occurred in correlation with a relatively high average temperature of 22.0˚C, nearly as high as the season's maximum average temperature (22.8˚C during Sept 20th to 26th; epiweek 38.5). Additionally, this abundance peak was recorded alongside a relatively low average precipitation value of only 1.2mm; as such, precipitation may not have as much of an effect on *Culicoides* numbers as previously thought (Table 5.8).

Once again, two separate instances of abundance spikes in *Bezzia* were observed. This observation confirms that *Bezzia* appears to exhibit a secondary emergence of adults at some point late in the summer season, as an additional population increase occurred in late August during every year of sampling. The second observed spike for each year was always lower in abundance than that of the primary generation.

A sharp drop in *Probezzia* was observed after the genus' abundance spike, coinciding with a rise in *Bezzia*. This may lend further support to the possibility that *Bezzia* and *Probezzia* exhibit direct competition or predation upon one another, as the arrival of high numbers of *Bezzia* appears to have driven *Probezzia* abundance downward.

5.4.2 Discussion of Redundancy Analysis (RDA)

Collection year (year) and amount of precipitation (PRECIP) did not contribute to overall pattern of variance, as shown by their extremely small arrow vectors. Temperature (TEMP) and month of collection (month) were strong contributors to the pattern of variance, illustrated by their large arrow vectors. Relative humidity (HUM), average pressure (AVGPRS), and wind speed (WINDSPD) displayed moderate contribution to the pattern of variance.

Examination of the trends observed in Figure 5.7 reveal multiple patterns within the dataset. First and foremost, it should be noted that vector arrows representing precipitation (PRECIP) and year of capture (year) are very small, suggesting that those particular variables did not have much impact on the variance of the dataset, and thus exerted little effect on the abundance of genera. As such, these factors will not be discussed any further.

The most impactful climate variables with respect to biting midge abundance and activity appear to be temperature (TEMP) and month of capture (month), as their representative vector arrows are the longest. This suggests that these particular variables accounted for the biggest impact on variation in the data. With respect to direct relation, temperature and month do not appear to be related to one another as indicated by the angle of approximately 90 degrees between their vectors. However, a great number of the vector arrows for Ceratopogonidae genera show a positive correlation with temperature, with genus *Ceratopogon* Meigen, 1803 (CERA) being the only exception. This particular genus is shown to have a very slight negative correlation with temperature, but this is likely an artifact of infrequent collection; only three *Ceratopogon* specimens were collected over the entire collection period, and so their relationship with climate variables is not likely to be accurate.

The data of all remaining genera, however, generated vectors with angles less than 90 degrees to the temperature vector, suggesting that they are at least somewhat positively correlated with ambient temperature. This establishes temperature as an important predictor variable for a number of collected genera, particularly those with a vector arrow existing very close to the vector arrow for temperature. For example, the arrow vector for genus *Palpomyia* Meigen, 1818 is directly overlapping with the vector for temperature, suggesting an extremely strong positive relationship. The genera *Culicoides* and *Probezzia* display only some correlation with temperature, though this may be skewed as a result of the frequent population spikes that these genera display (Figures 5.2 to 5.6). Some species of biting midges overwinter in an immature state and then emerge in the spring, leading to a dramatic increase in active, flying adult forms. Though this is slightly correlated with temperature, which affects rates of development, population spikes are also highly dependent on midge activity in the previous year, specifically with respect to egg deposition and overwintering success, particularly in the Canadian climate. Harsh winter seasons may contribute to the resultant observed abundances of various genera, including *Culicoides*. Therefore, examination of winter temperatures and conditions occurring prior to emergence of genera such as *Culicoides* may reveal factors that are more indicative of their success throughout the spring and summer.

Though month of capture had a large effect on overall variance, it was not directly correlated with any biting midge genera, as their patterns are more associated with the temperature attributed to the monthly progression throughout the year rather than the month itself. Therefore, variance brought about by month is not a result of the month alone, but rather seasonal climate patterns. Such additional variables may include relative humidity (HUM) and average pressure (AVGPRS), both of which were found to be positively correlated with month

and exhibited a moderate effect on variance of collection data in the RDA results. Interestingly, nearly all biting midge genera were negatively correlated with average pressure, at least to some degree; *Forcipomyia* Meigen, 1818 is the only exception to this trend, although the positive relationship exhibited by this genus is minimal. Various insects, including Diptera, have been shown to exhibit increased activity in low-pressure climate systems, primarily associated with the decrease in pressure that occurs before events such as thunderstorms (Dethier, 1957). It has been postulated that increased activity before a storm may allow biting insects to take a blood meal or perhaps mate before conditions become unfavourable, particularly when the length of such weather is uncertain. However, the exact reason for this behaviour is not yet known.

The remaining moderate contributor to variance is wind speed (WINDSPD). The RDA depicts a majority of biting midge vector arrows as negatively correlated with wind speed, with only two exceptions: *Ceratopogon* and *Culicoides*. It might be predicted that wind speed would have a negative effect on biting midge activity since ceratopogonids are extremely small insects and are not strong fliers. Biting midges are able to be carried over long distances with strong wind gusts, as they are unable to fly against them. It has been suggested that even relatively low wind speeds are able to disrupt biting midge flight patterns (Ducheyne *et al*., 2007). As a result, adult midges tend to seek shelter and remain out of strong wind currents, reducing their activity during the daytime and instead exhibiting crepuscular tendencies. The genera displaying slight positive correlation with wind speed (*Culicoides* and *Ceratopogon*) are likely still affected in the same way, as their small body size (even relative to the body sizes of other biting midge genera) would be unlikely to withstand strong wind gusts. However, as mentioned during discussion about the effects of temperature on midge populations, only 3 *Ceratopogon* specimens were collected in total, and so the dataset for that genus is unlikely to be representative of its true

habits. Additionally, *Culicoides* population spikes may result in more captures of individuals even during times of high wind speed, as they are swept into the aerial plankton which is sampled by the Rothamsted trap.

The amount of precipitation did not have a strong effect of the variance of the system. Insects generally take shelter during bouts of rain and thunderstorms, and so it was deemed likely that there would be lower collection rates of biting midges associated with high precipitation; however, this was not the case, according to RDA analysis, as evidenced by the nearly negligible arrow vector for precipitation. While it is possible that this relationship is truly representative of the effect of precipitation on biting midge collection, there may also be another explanation as a result of data inaccuracy. It was noted in the methods for this chapter that consistent and reliable precipitation data was not available for St. Catharines, and so recordings from the proximal locality of Vineland were used instead. It could be that this amount of geographical distance between recorded precipitation values and the actual trap (~12.75 km) was enough to significantly skew the results. Therefore, it would be interesting to repeat this type of data collection and establish a method of precipitation measurement in the exact location of the Rothamsted trap to observe a more accurate relationship.

5.5 Conclusions

Analysis of *Bezzia*, *Culicoides*, and *Probezzia* abundances captured by the Rothamsted trap in the summer seasons revealed numerous trends that may be used to assess ideal timeframes for capturing and observing these ceratopogonids. *Bezzia* exhibited their highest abundances in approximately mid-July, with a second smaller population spike occurring in late

August. RDA results indicated that *Bezzia* activity was highly correlated with temperature, suggesting that high temperature values directly affect the abundance of this genus. *Culicoides* trends were less consistent, with population spikes occurring between late May and mid- to late July; this genus was also not found to be highly associated with recorded temperature values. These findings suggest that other factors influence *Culicoides* mass emergence, and it is possible that winter conditions are contributors to these patterns. *Probezzia* are most likely to be captured between mid-June and early July, and may exhibit competition interactions with *Bezzia*. RDA analysis found that nearly all collected genera exhibited moderate to strong association with recorded temperature. Wind speed and average pressure were found to be moderate contributors to reduced biting midge activity.

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Visual Key to Nearctic Genera of Biting Midges (Diptera: Ceratopogonidae)

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Click to begin

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Introduction

Ceratopogonidae, also known as biting midges, no-see-ums, or punkies, are predaceous dipteran insects with females known for their habit of biting vertebrate and invertebrate hosts to take blood meals, though only members of a few North American genera (*Culicoides*, *Forcipomyia*, *Leptoconops*) display vertebrate-feeding behaviour. The remaining biting midge species instead feed upon invertebrate hosts such as mayflies and families of Nematoceran Diptera, extracting nutritious haemolymph. Though the vertebrate-feeding genus *Culicoides* is often noted for its small size and ability to move through screen doors and windows in search of a host, there are a number of genera, such as *Sphaeromias* and *Nilobezzia*, with species that often reach a size comparable to North American mosquitoes in their adult phase.

In general, ceratopogonids display a life cycle involving an aquatic or semi-aquatic larval stage, though species in subfamily Forcipomyiinae may have terrestrial crawling larval forms that feed on plant and fungal material. Eggs are deposited near the shoreline of water or in moist soil, where they hatch to first instar larvae and begin to feed upon microorganisms and diatoms in the medium. Larvae progress through four instar stages before pupation; though Forcipomyiinae pupal forms are immobile, pupae of many other genera are capable of slight movements, and some may retain the ability to swim. Aquatic and semi-aquatic pupae rest near the surface of the water column where the insect emerges as the adult form. Females usually require a haemolymph or blood meal to mature their eggs, but some species are known to be autogenous and may deposit their first eggs without requirement for additional nutrition.

Although some vertebrate-biting species are important as disease vectors of animal viruses such as Bluetongue and Epizootic Hemorrhagic Disease, Ceratopogonidae remains a relatively understudied family, and as such it is difficult for taxonomists and non-taxonomists alike to reliably identify species. Additionally, the physical appearance and structures of biting midges can be highly variable between genera. This key adapts the overall structure of the Ceratopogonidae genus key found in the *Manual of Nearctic Diptera Vol. 1* (Downes and Wirth, 1981) and supports key characters with high definition specimen photography. Specimens were either captured using the Brock University Rothamsted trap or they were borrowed from insect collections at other facilities, including the Canadian National Collection of Insects, Arachnids, and Nematodes (Ottawa, ON) and the National Museum of Natural History (Washington, DC).

The purpose of this photographic key is to aid in the identification of biting midges to at least genus level, although genera containing only one recognized species provide information to the species level. A glossary of commonly used terms for identification is provided to help further with accurate identification.

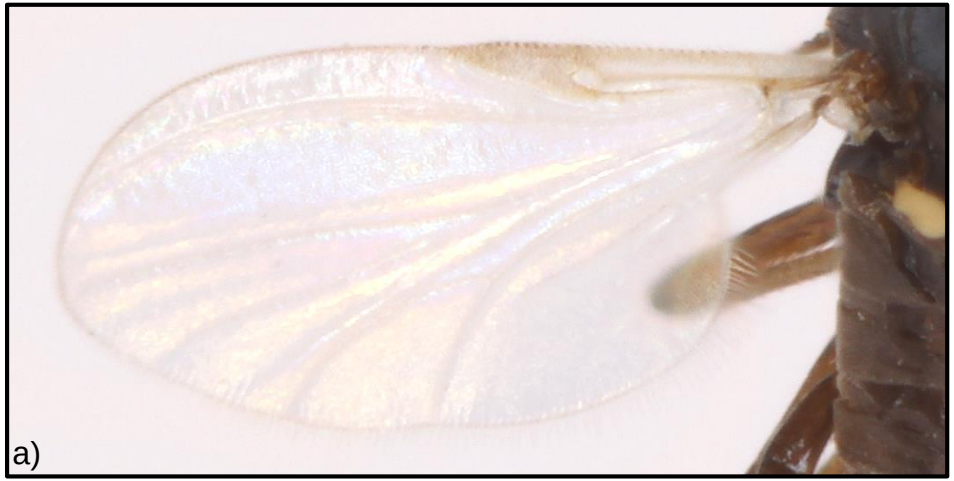
Checklist of Genera and Species Listed in Key

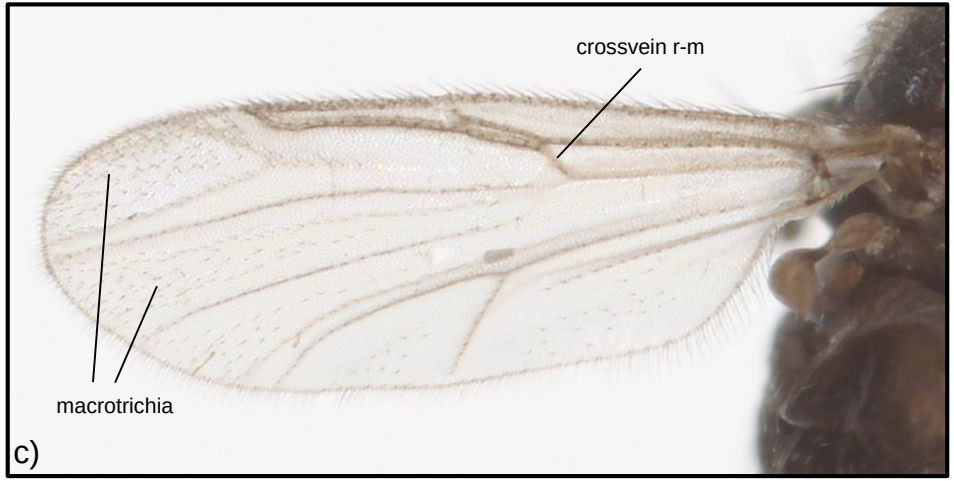
Subfamily Ceratopogoninae
- *Alluaudomyia* Kieffer, 1913
- *Bezzia* Kieffer, 1899
- *Brachypogon* Kieffer, 1899
  - *Brachypogon* Kieffer, 1899
  - *Isohelea* Kieffer, 1917
- *Ceratoculicoides* Wirth & Ratanaworabhan, 1971
- *Ceratopogon* Meigen, 1803
- *Clinohelea* Kieffer, 1917
- *Culicoides* Latreille, 1809
- *Echinohelea* Macfie, 1940
  - *lanei* Wirth, 1951
- *Heteromyia* Say, 1825
- *Jenkinshelea* Macfie, 1934
- *Johannsenomyia* Malloch, 1915
- *Macropeza* Meigen, 1818
  - *blantoni* Wirth & Ratanaworabhan, 1972
- *Mallochohelea* Wirth, 1962
- *Monohelea* Kieffer, 1917
- *Neurobezzia* Wirth & Ratanaworabhan, 1972
  - *granulosa* Wirth, 1952
- *Neurohelea* Kieffer, 1925
- *Nilobezzia* Kieffer, 1921
- *Pachyhelea* Wirth, 1959
  - *pachymera* (Williston, 1900)
- *Palpomyia* Meigen, 1818
- *Parabezzia* Malloch, 1915
- *Paradasyhelea* Macfie, 1940
- *Paryphoconus* Enderlein, 1912
  - *sonorensis* Wirth & Ratanaworabhan, 1972
- *Pellucidomyia* Macfie, 1939
  - *wirthi* (Lane, 1956)
- *Phaenobezzia* Haeselbarth, 1965
- *Probezzia* Kieffer, 1906
- *Rhynchohelea* Wirth & Blanton, 1970
  - *monilicornis* Wirth & Blanton, 1970
- *Serromyia* Meigen, 1818
- *Sphaeromias* Curtis, 1829
- *Stenoxenus* Coquillett, 1899
  - *johnsoni* Coquillett, 1899
- *Stilobezzia* Kieffer, 1911
  - *Eukraiohelea* Ingram & Macfie, 1921
    - *elegantula* (Johannsen, 1907)

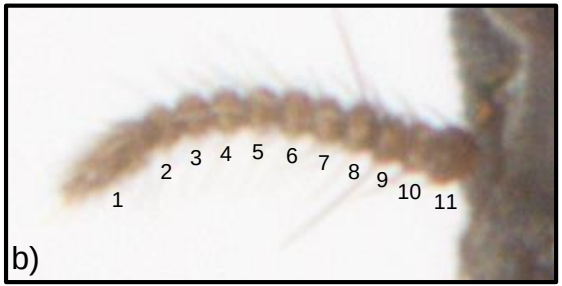
Subfamily Dasyheleinae
- *Dasyhelea* Kieffer, 1911

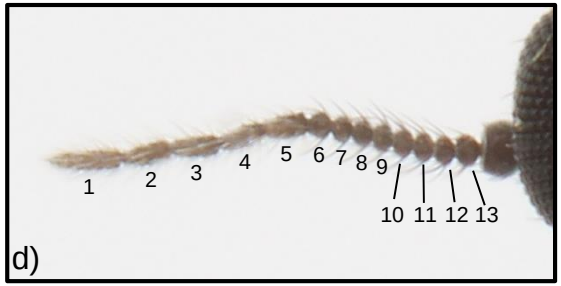
Subfamily Forcipomyiinae
- *Atrichopogon* Kieffer, 1906
- *Forcipomyia* Meigen, 1818

Subfamily Leptoconopinae
- *Leptoconops* Skuse, 1889

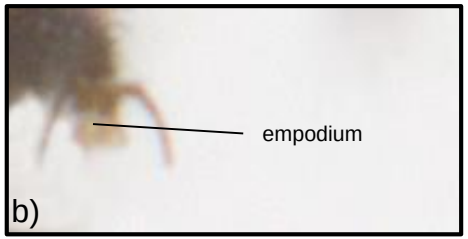


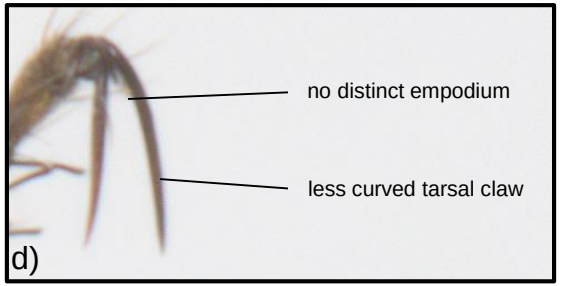


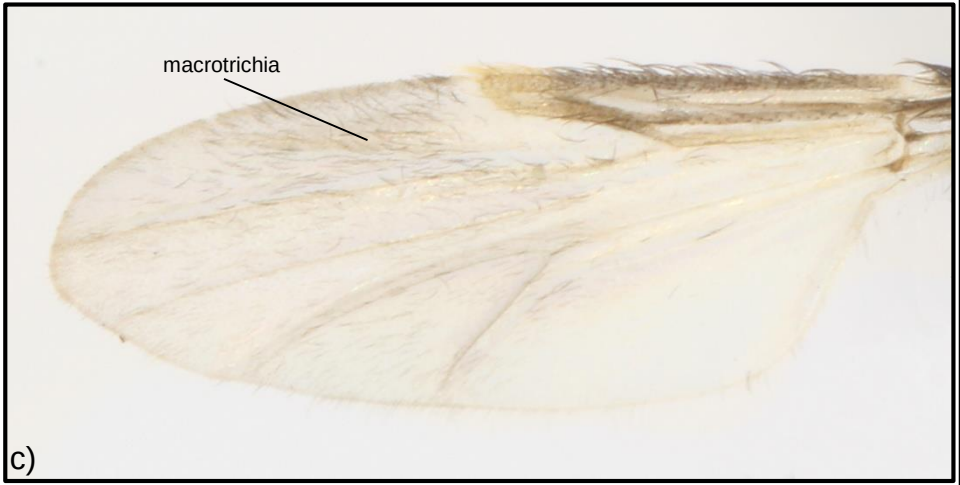




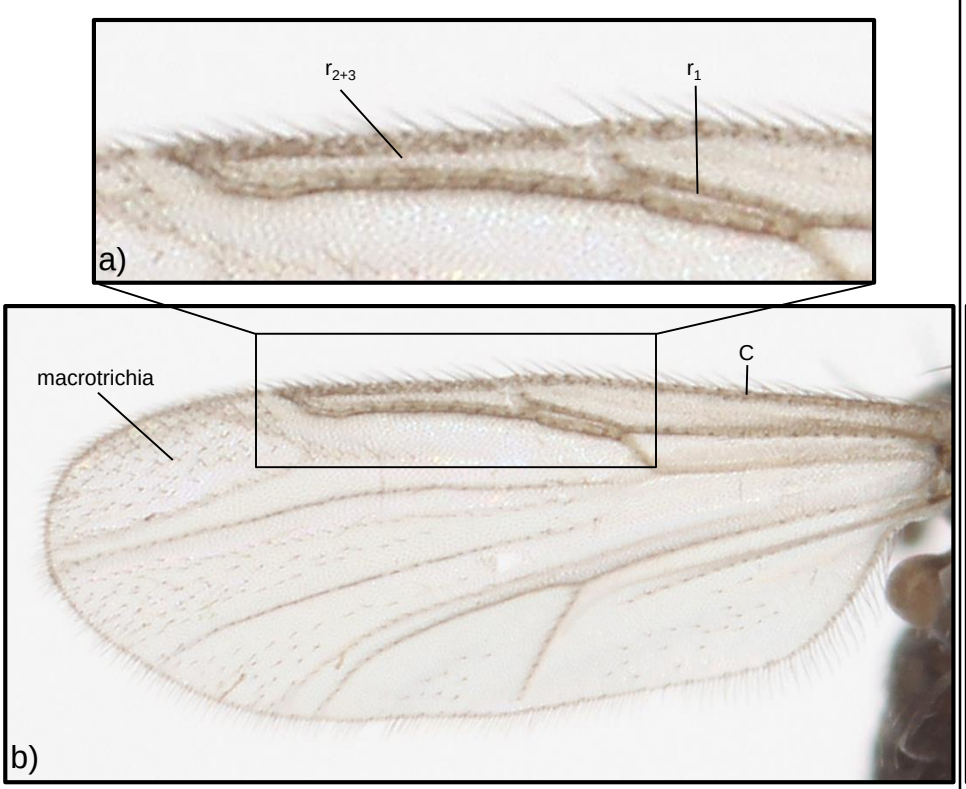
1	Wing with crossvein r-m absent, and without macrotrichia (a); female antenna with 11-12 flagellomeres (b).	LEPTOCONOPINAE *Leptoconops*
-	Wing not as in *Leptoconops*; crossvein r-m present between radial and medial veins, and usually with macrotrichia or microtrichia (c); female antenna with 13 flagellomeres (d).	2

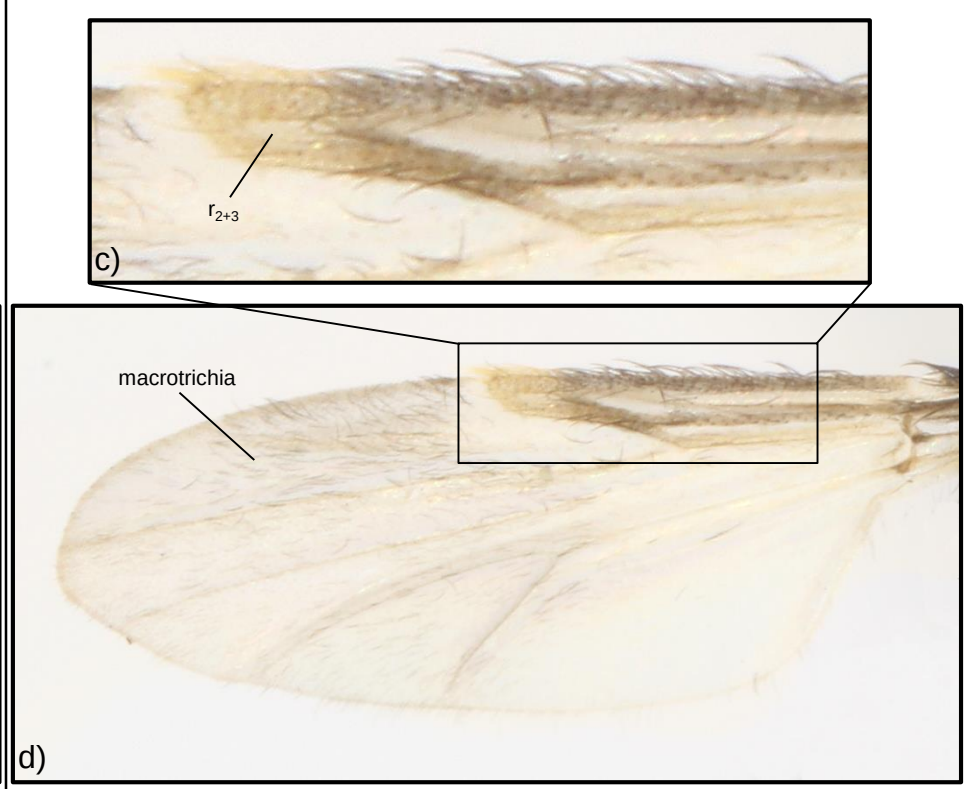




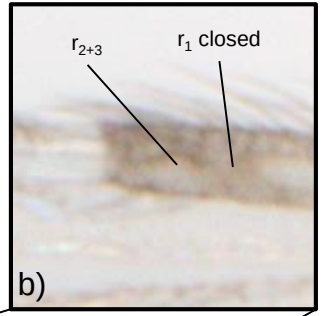


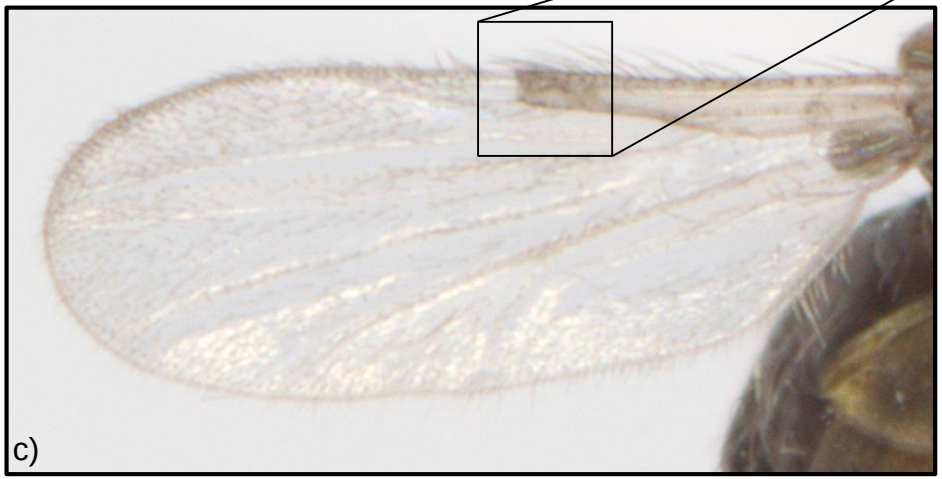
2 (1)	Tarsal claws strongly curved, arcuate (a) and with empodia strongly developed, at least in female (b); wing usually with numerous macrotrichia (c).	FORCIPOMYIINAE...3
-	Tarsal claws not strongly curved, with empodia small or vestigial (d); wing usually with macrotrichia less abundant or absent (e).	4

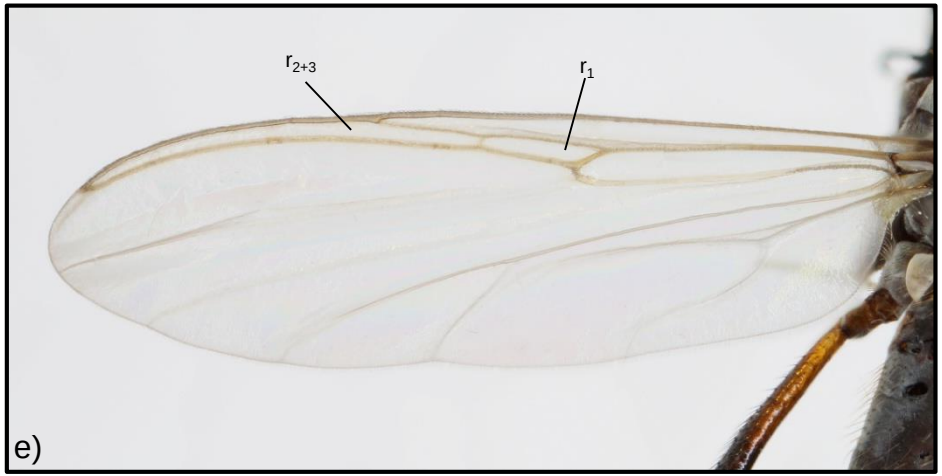




3 (2)	Cell r_{2+3} approximately twice as long as cell r_1 (a); costal vein, C, reaching well beyond middle of wing; macrotrichia, when present, scattered (b).	*Atrichopogon*
-	Cell r_{2+3} usually short, but if long then distinctly narrow (c); C length various; macrotrichia abundant and covering most of the wing (d).	*Forcipomyia*

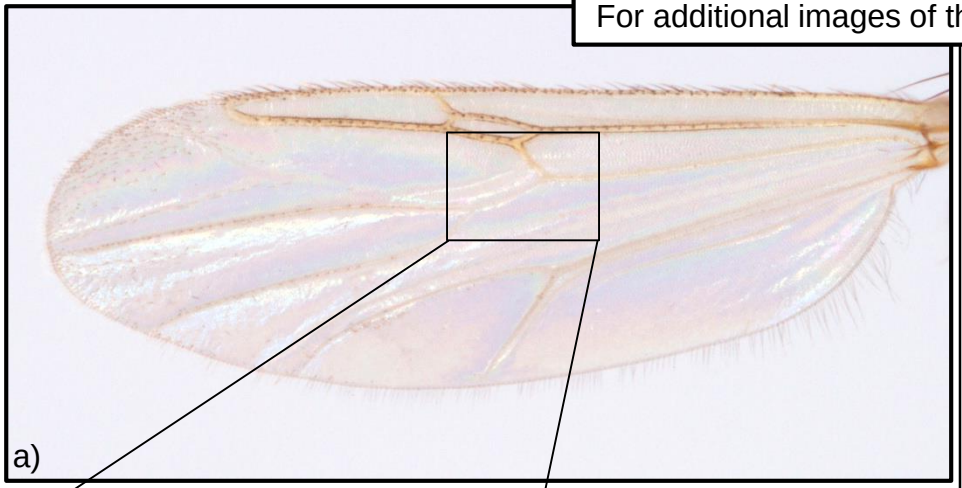


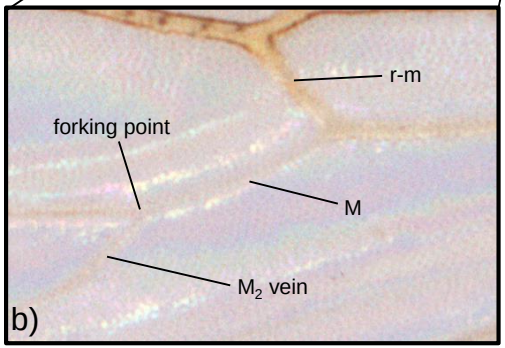


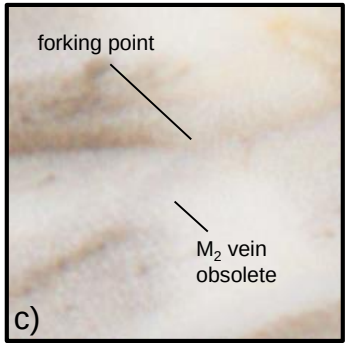


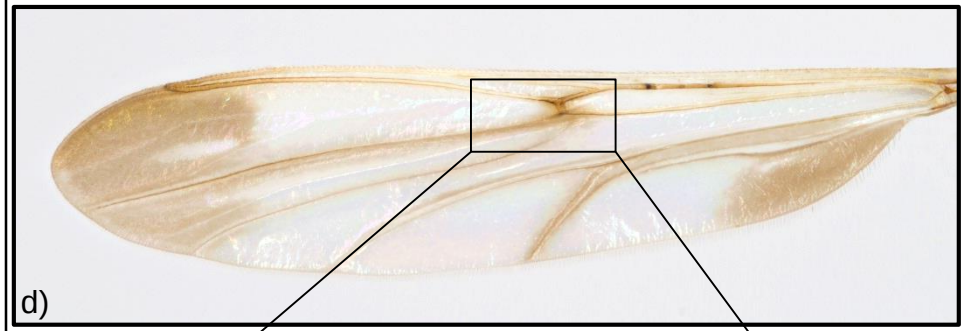
4 (2)	Antennal flagellomeres sculptured (a); cell r_1 nearly or completely closed (b); cell r_{2+3} roughly square-ended, frequently ending at or before middle of wing (c).	DASYHELEINAE *Dasyhelea*
-	Antennal flagellomeres not sculptured (d); cell r_1, cell r_{2+3}, or both well developed; cell r_{2+3} not square-ended, often ending beyond middle of wing [except in one genus, *Paradasyhelea*] *(*e)	CERATOPOGONINAE ……..5

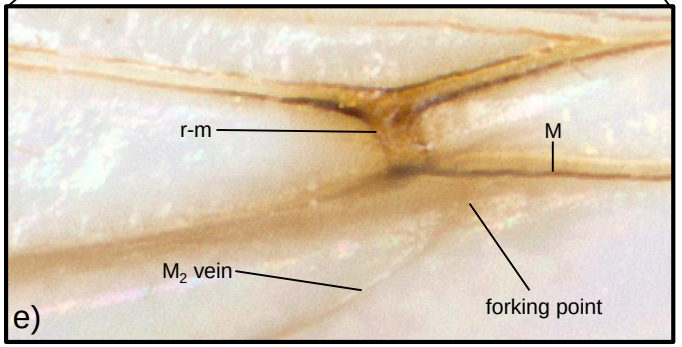
For additional images of these characters, click here.



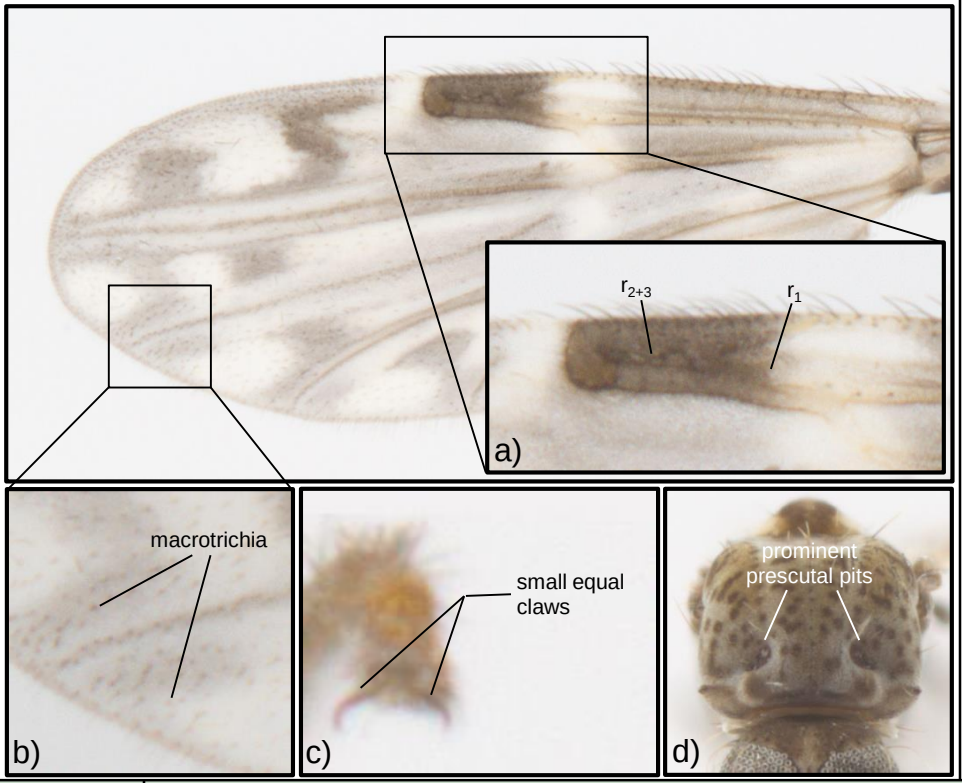


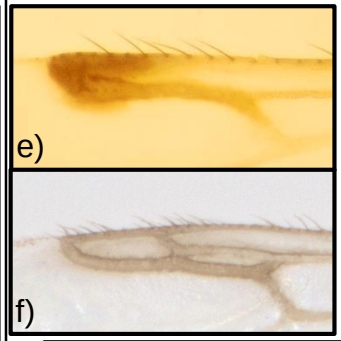


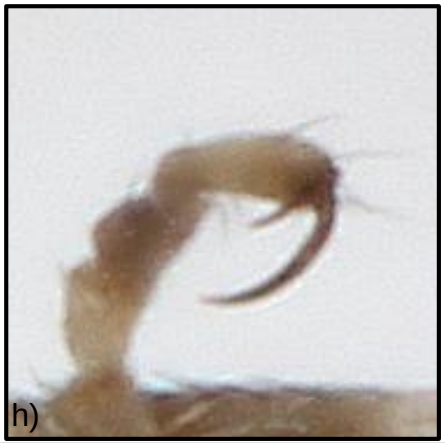


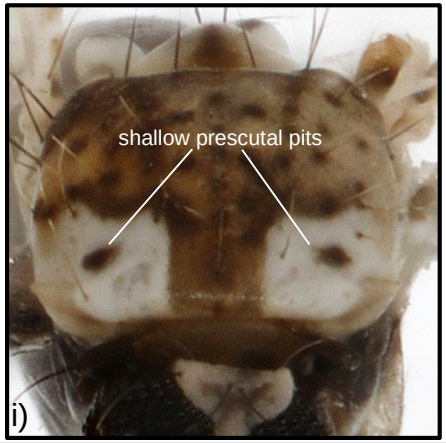


5 (<u>4</u>)	Medial vein, M, forking beyond crossvein r-m, except in spinose-legged genus *Echinohelea* with M forking just at r-m (a, b); second medial vein, M_2, sometimes obsolete basally (c).	<u>6</u>
-	M forking at or before crossvein r-m, with M_2 **almost** always complete (d, e).	<u>19</u>

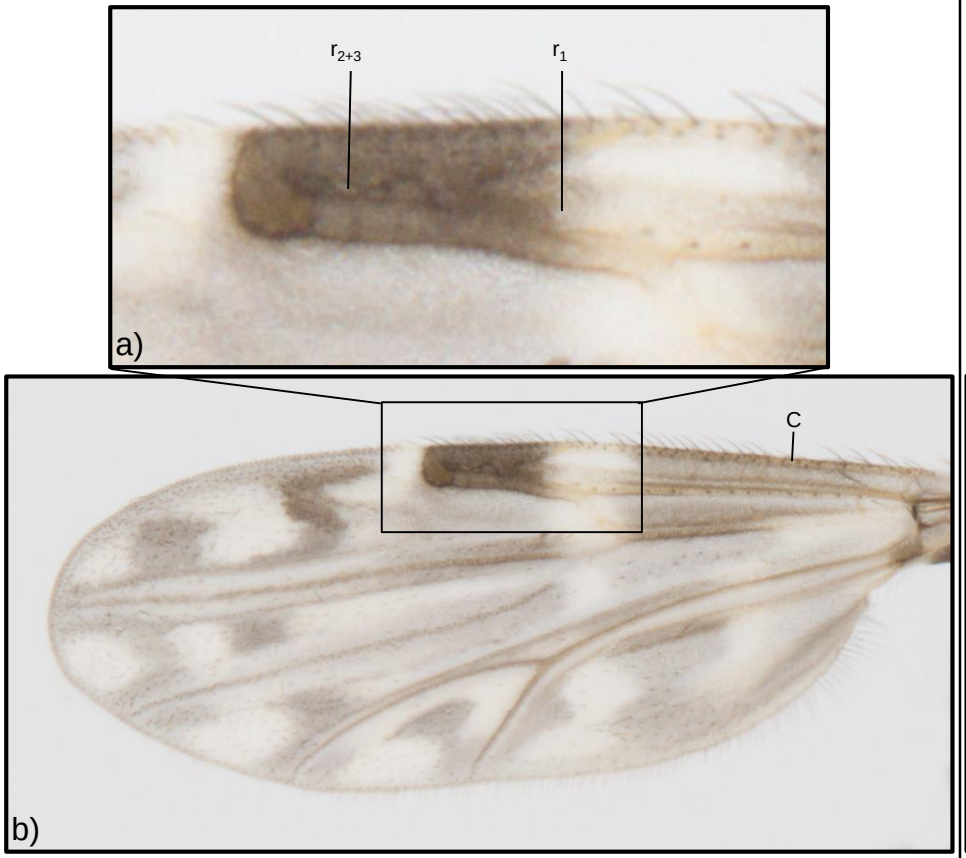


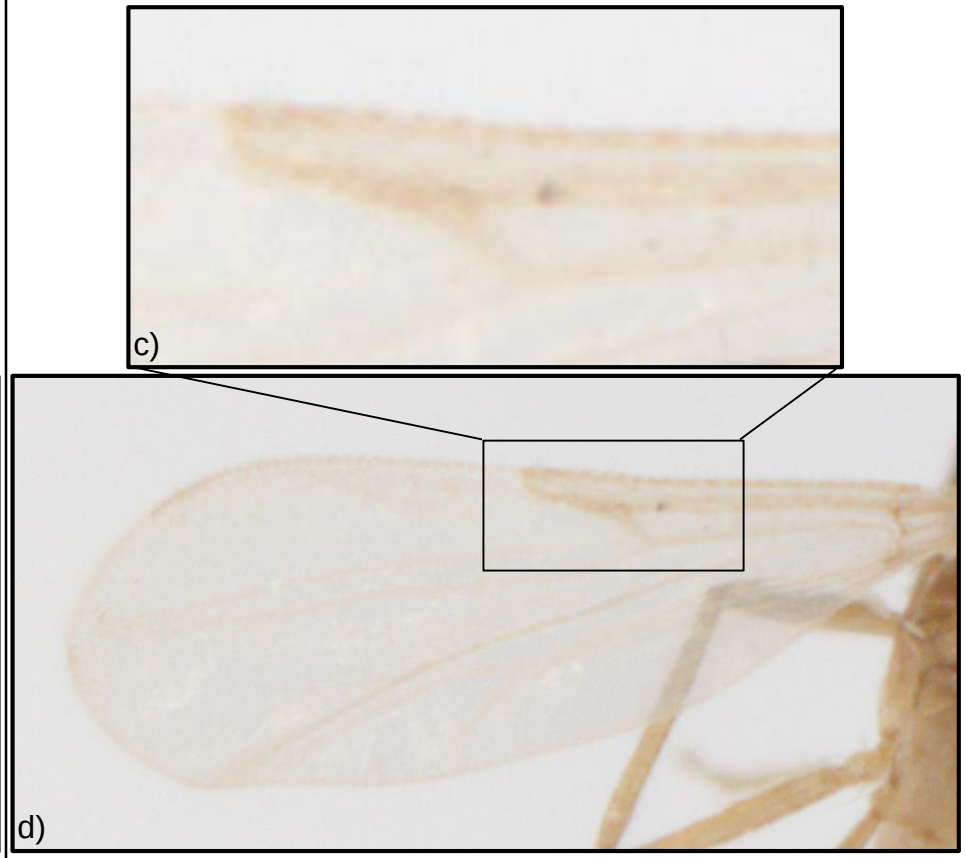




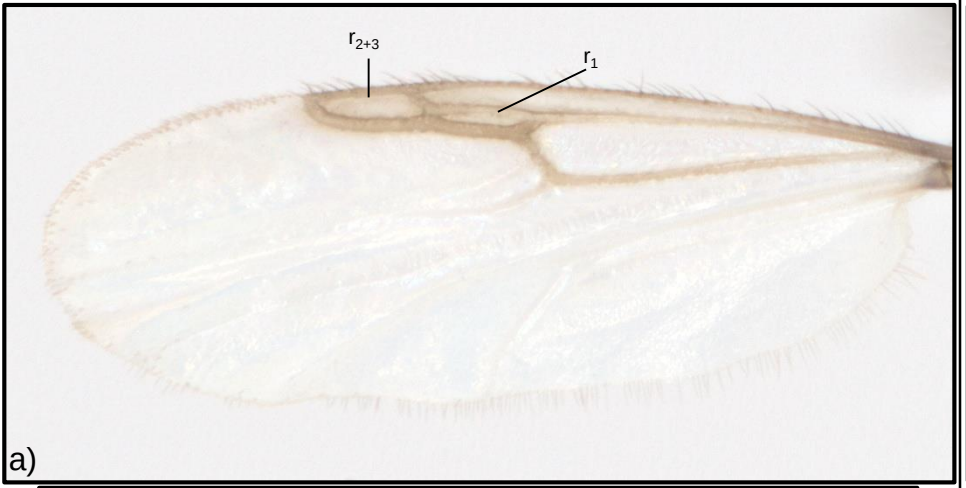


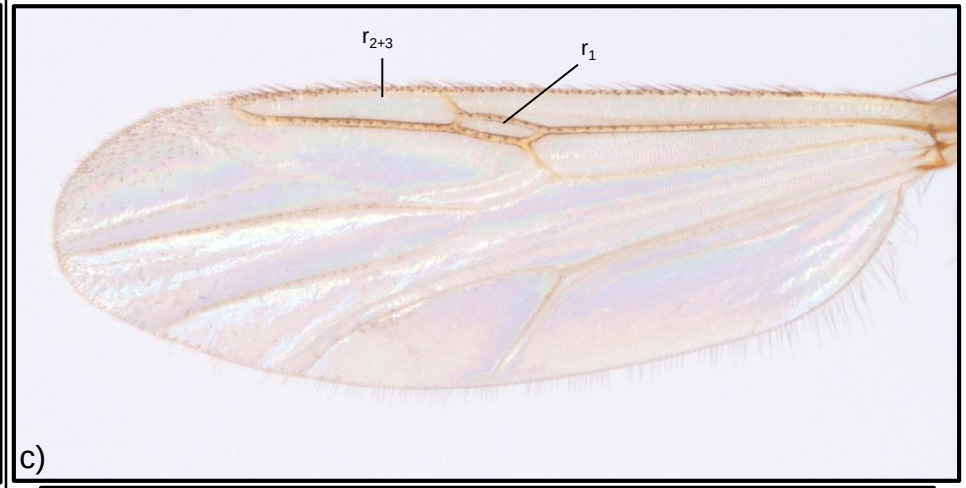
6 (5)	Cells r_1 and r_{2+3} usually well-developed and similar in size (a); wing macrotrichia usually abundant (b); claws small and equal (c); anterior region of thorax with prominent prescutal pits (d).	CULICOIDINI……7
-	Cell r_1 and, rarely, r_{2+3} sometimes closed or lost (e) **OR** cell r_{2+3} larger than r_1 (f); wing macrotrichia usually less numerous, sometimes absent (g); female claws usually larger, equal, or unequal (h); prescutal pits small or absent (i).	8



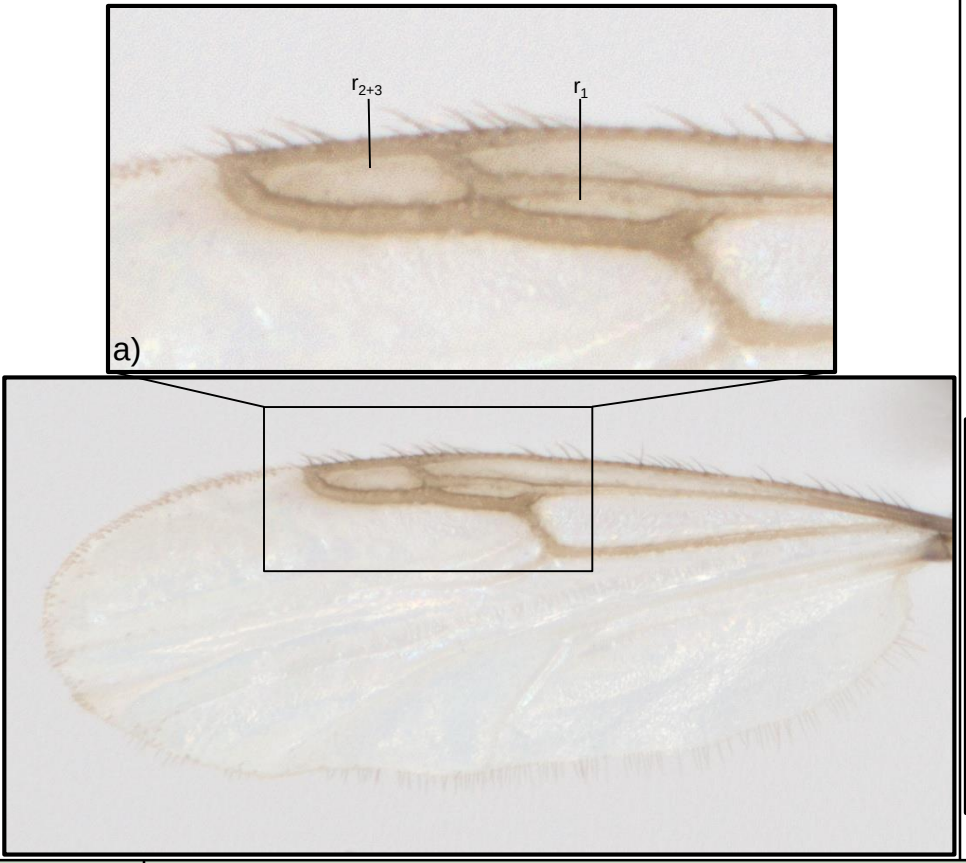


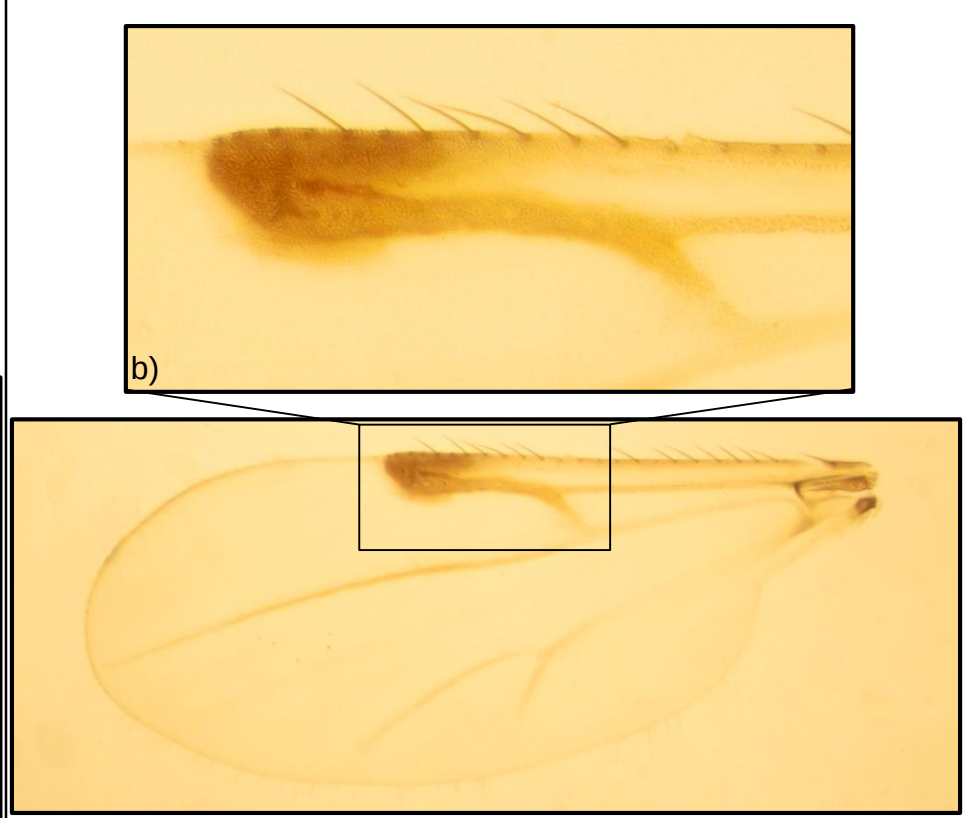
7 (6)	Cells r_1 and r_{2+3} usually well-developed (a); costal vein, C, extending past middle of wing; wing commonly decorated with pale or darkened spots (b).	*Culicoides*
-	Cells r_1 and r_{2+3} obliterated (c); C short and not reaching middle of wing; wing without spotted patterns (d).	*Paradasyhelea olympiae*



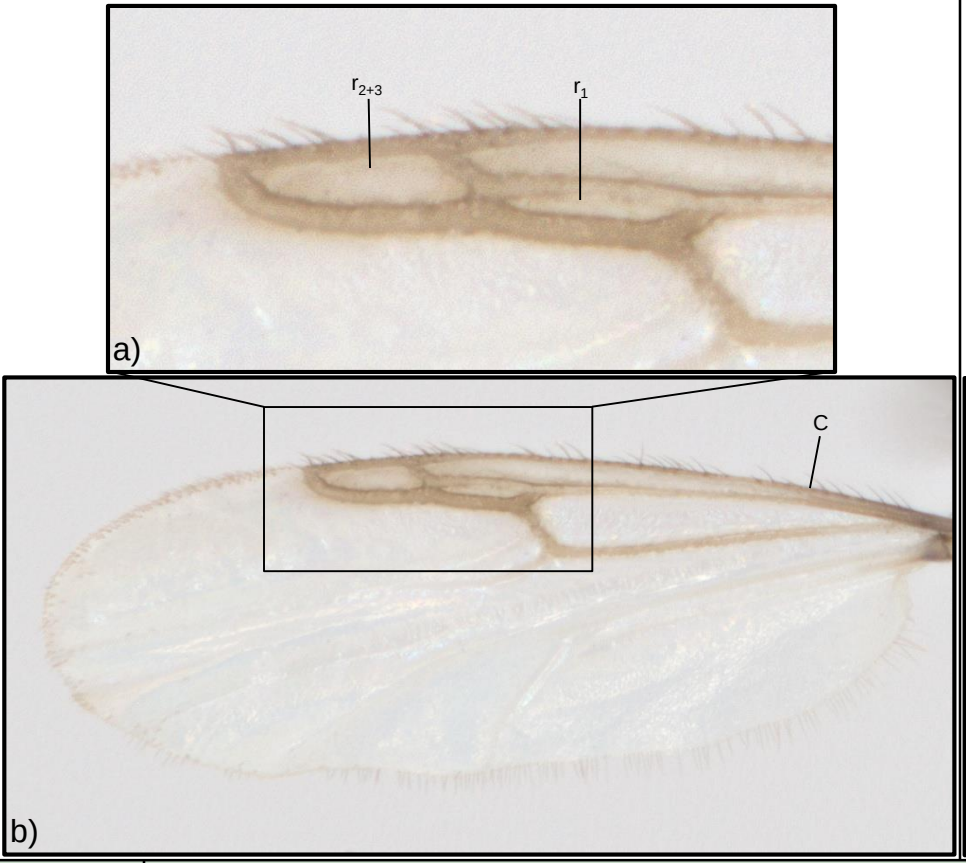


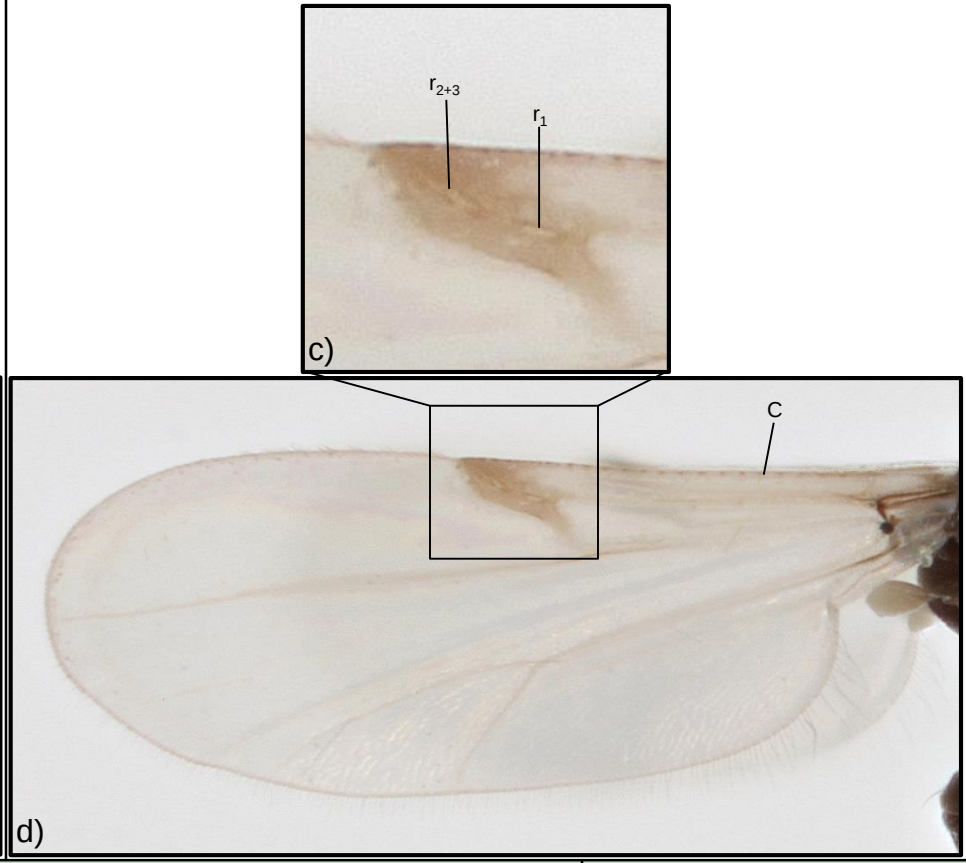
8 (6)	Cell r_{2+3} small, **at most** slightly longer than r_1, one or both cells sometimes lost; wing often milky (a) or, in most *Alluaudomyia*, with black spots or streaks (b); eye often pubescent (not pictured).	CERATOPOGONINI…9
-	Cells r_{2+3} long, often much longer than r_1; r_1 sometimes lost; wing not milky; often hyaline (c) or with dark patterning (d); eye usually bare (not pictured).	STILOBEZZIINI……..14



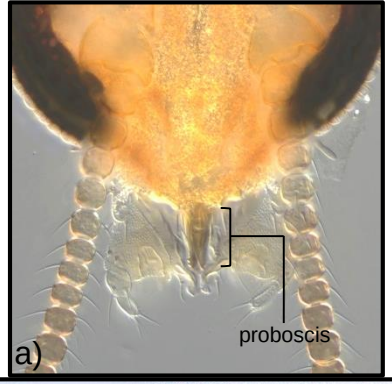


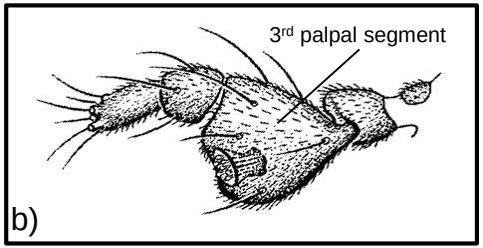
9 (8)	Cells r_{2+3} and cell r_1 complete (a).	10
-	One or both of cells r_{2+3} and r_1 obsolete (b).	11



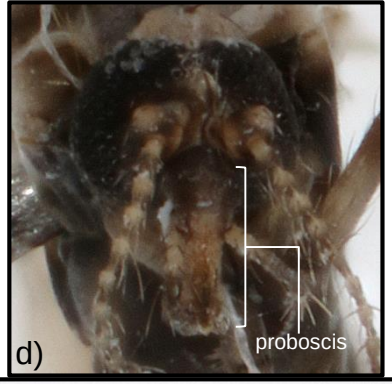


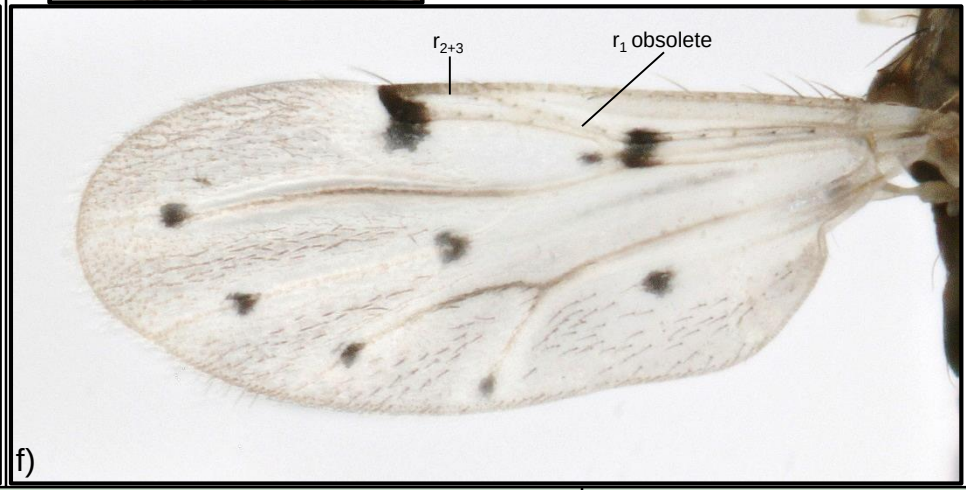
10 (9)	Cells r_1 and r_{2+3} elongate (a); costal vein, C, extending well past middle of wing (b).	*Ceratopogon*
-	Cells r_1 and r_{2+3} short with thickened adjacent veins (c); C extending to approximately middle of wing (d).	some *Brachypogon (Isohelea)*



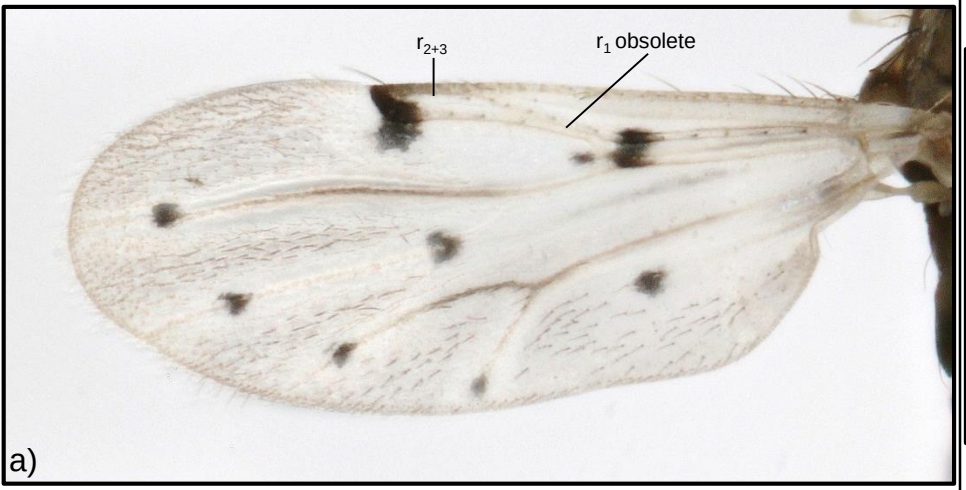


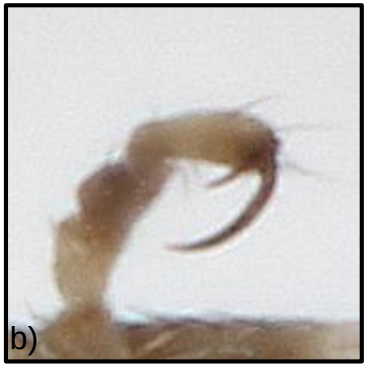
*Photograph not available. Image from Manual of Nearctic Diptera Vol. 1 (Downes and Wirth, 1981) with permission of Diptera unit at the Canadian National Collection of Insects, Arachnids, and Nematodes.

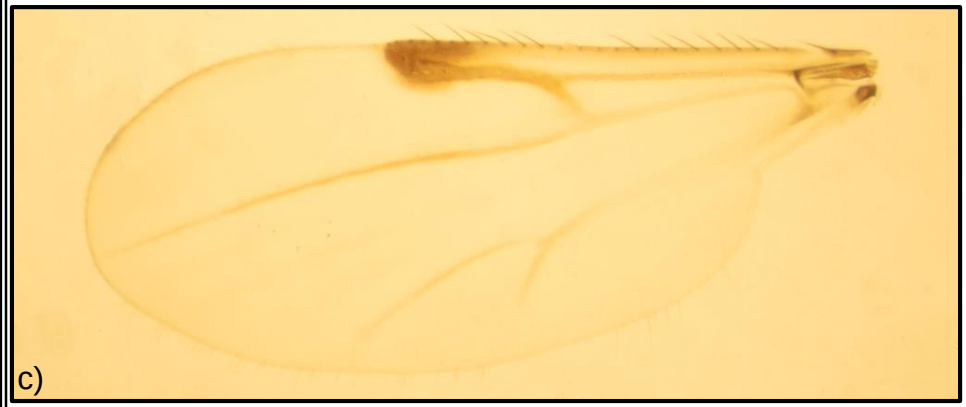


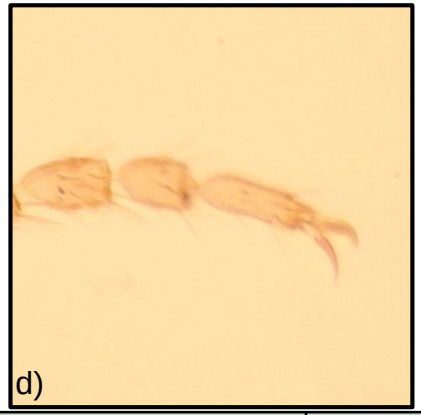


11 (<u>10</u>)	Proboscis stout, truncate (a); palpus stout, with 3rd segment broadened (b); cells r_1 and r_{2+3} absent; M_1 obsolete distally, M_2 absent (c). Very rare; known from California and Florida.	*Rhynchohelea monilicornis*
-	Proboscis longer, not stout or truncate (d); palpus slender (e); cell r_1 obsolete, and cell r_{2+3} developed **or** obsolete (f).	<u>12</u>

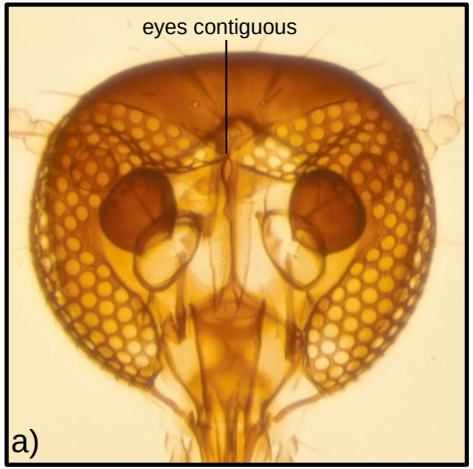


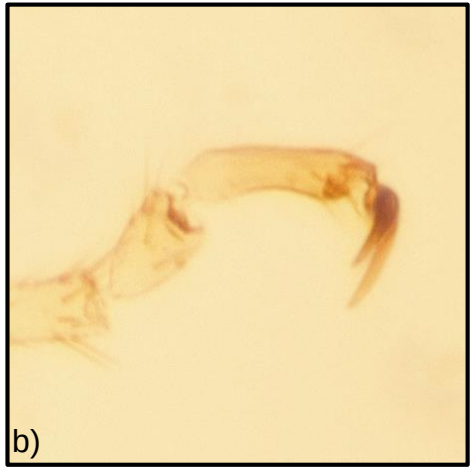


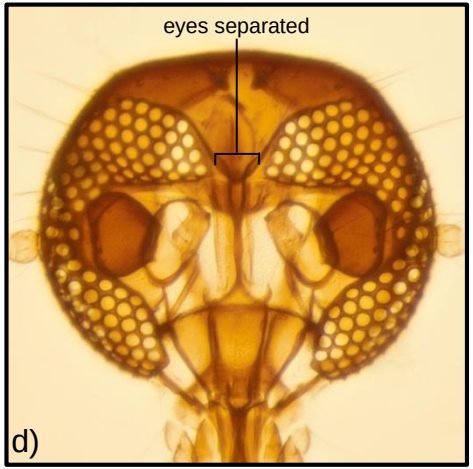


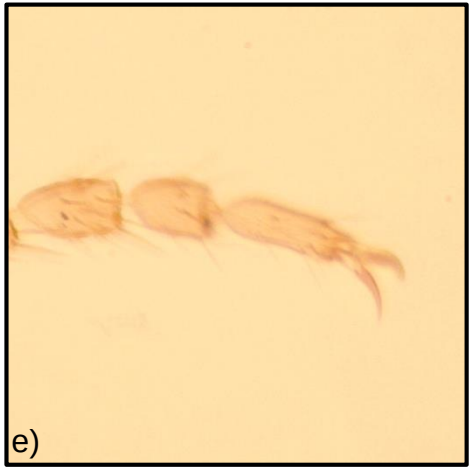


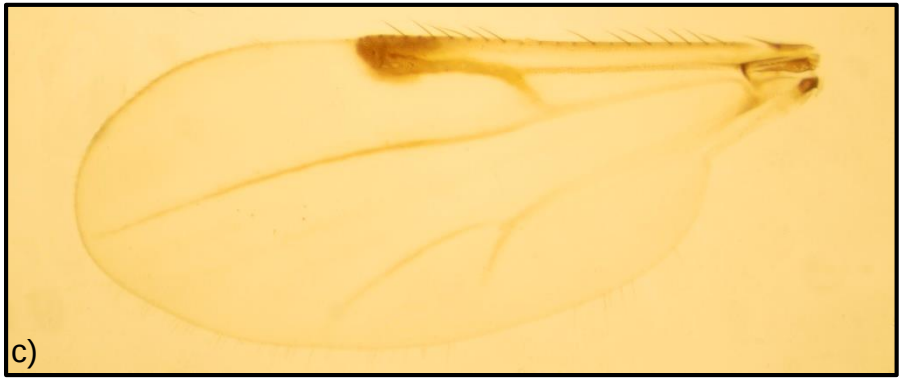
12 (<u>11</u>)	Cell r_1 obsolete, cell r_{2+3} well-developed; wing often with black spots or streaks (a); hind claws of female unequal (b).	*Alluaudomyia*
-	Cell r_1 obsolete, cell r_{2+3} small or also obsolete; wing with or without pattern (c); female claws equal on all legs (d).	<u>13</u>

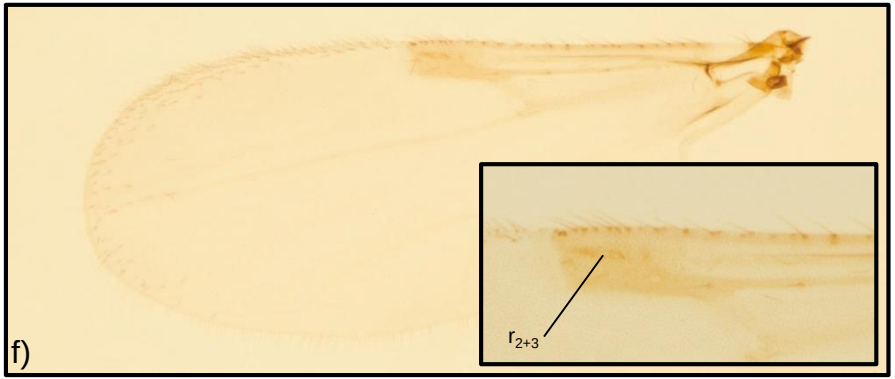




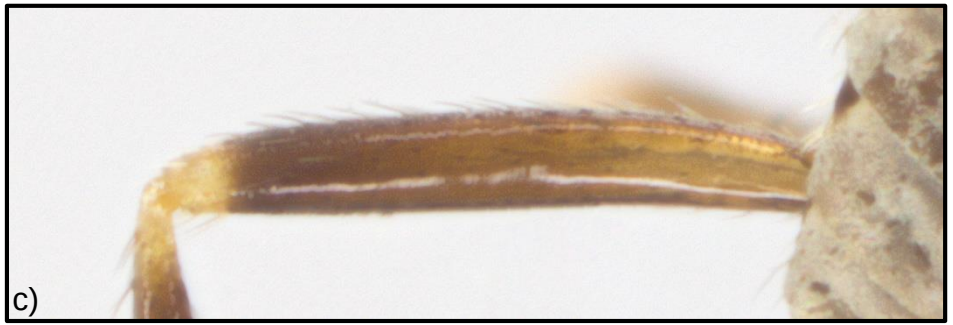




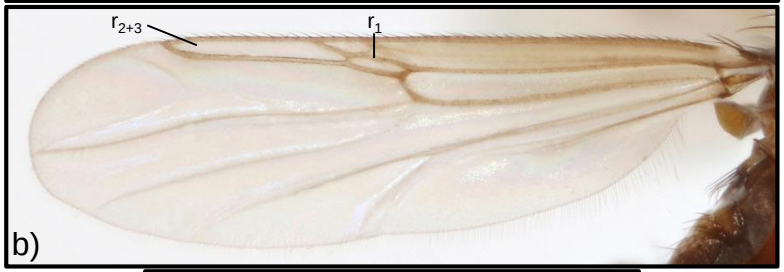


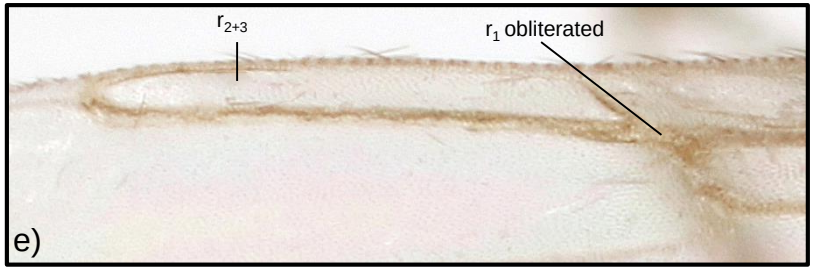


13 (<u>12</u>)	Eyes contiguous, meeting at top of head (a); female claws somewhat to moderately enlarged (b); cells r_1 and r_{2+3} obsolete (c).	*Brachypogon (Brachypogon)*
-	Eyes separated at top of head (d); female claws small on at least hindleg (e); cell r_1 obsolete, but cell r_{2+3} present and small (f).	*Ceratoculicoides*

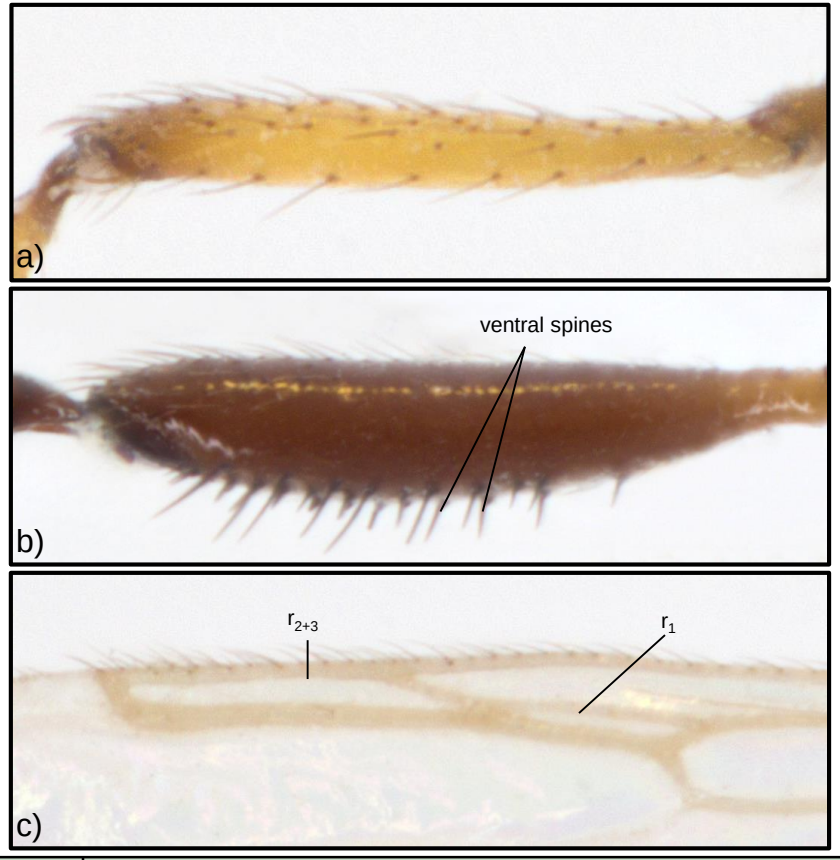


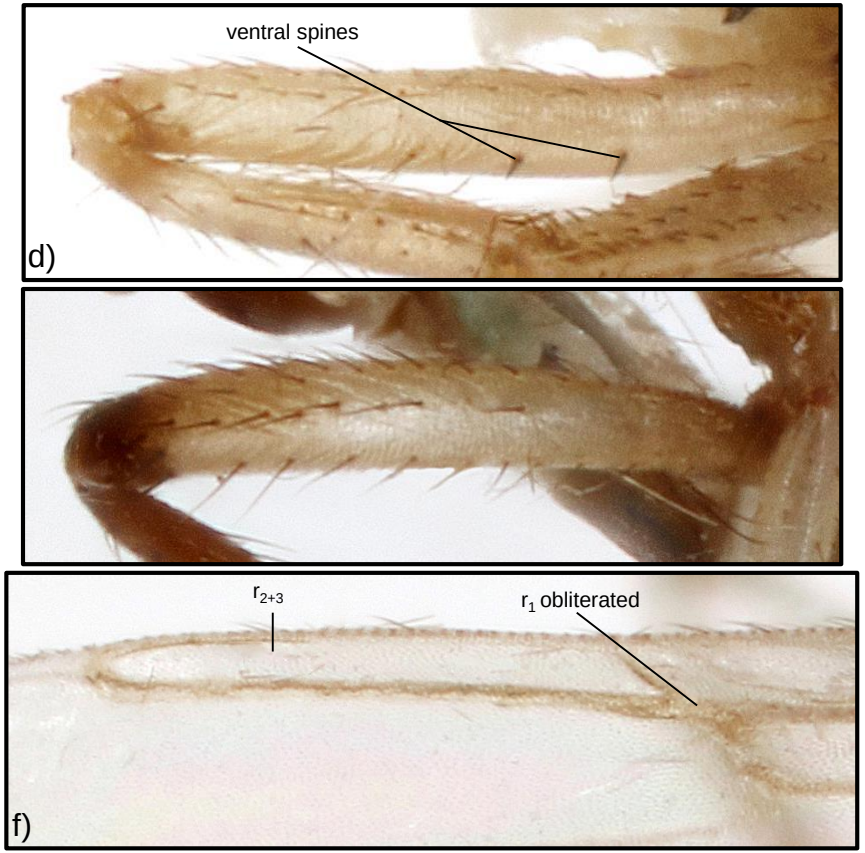
14 (8)	At least one pair of femora armed with one or more ventral spines (a, b)	15
-	No femora armed with ventral spines (c).	17



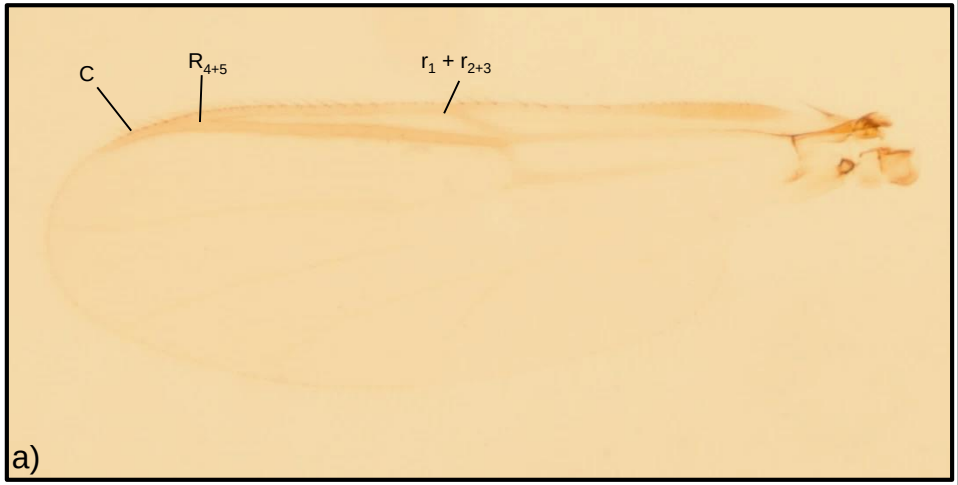


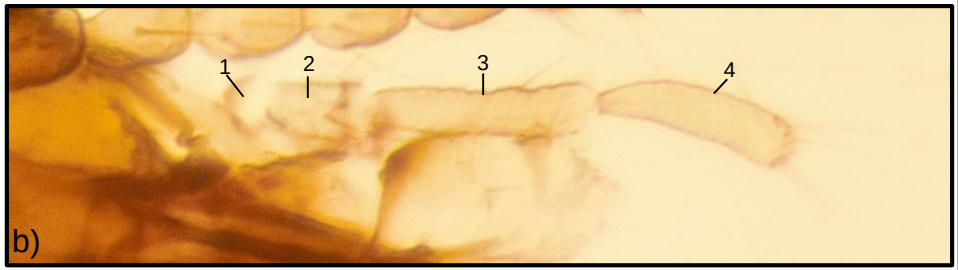
15 (14)	All femora with numerous spines, at least in male; spines not confined to ventral surface (a); cells r_1 and r_{2+3} well-developed (b); male antenna **not** plumose; last 5 flagellomeres elongated (c).	*Echinohelea lanei*
-	Only fore or hind femur with ventral spines (d); cell r_1 or r_{2+3} sometimes lost (e); male antenna plumose (f).	16

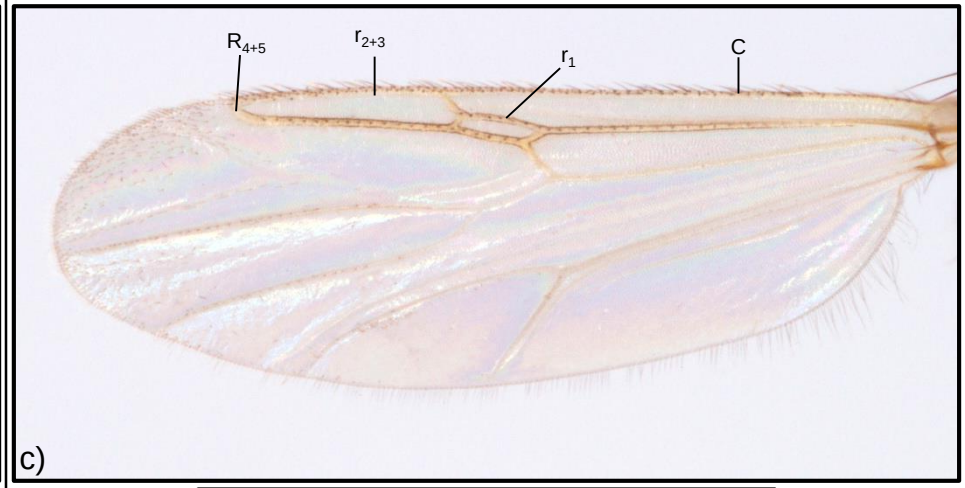


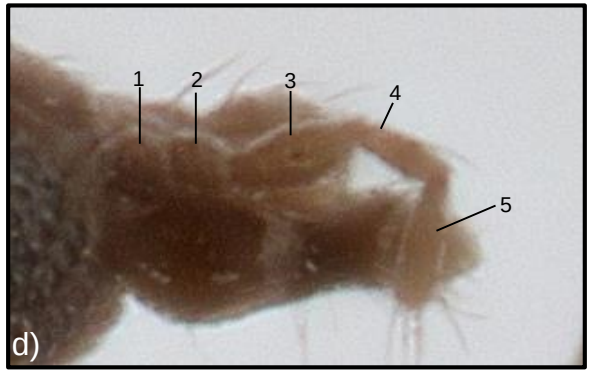


16 (<u>15</u>)	Fore femur unarmed (a); hind femur swollen, arcuate, and armed with ventral spines (b); cell r_1 and r_{2+3} well-developed (c).	*Serromyia*
-	Fore femur with 1-2 ventral spines (d); hind femur unarmed and not swollen (e); cell r_1 obliterated (f).	*Stilobezzia (Eukraiohelea)*

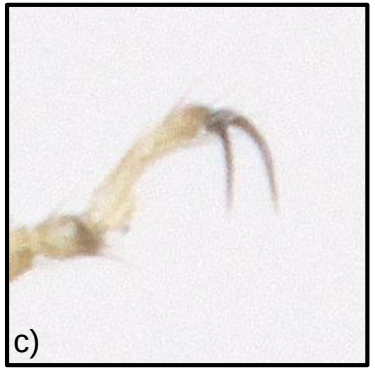


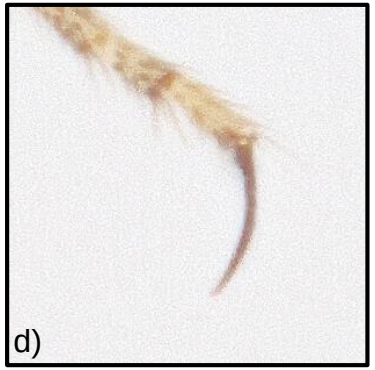


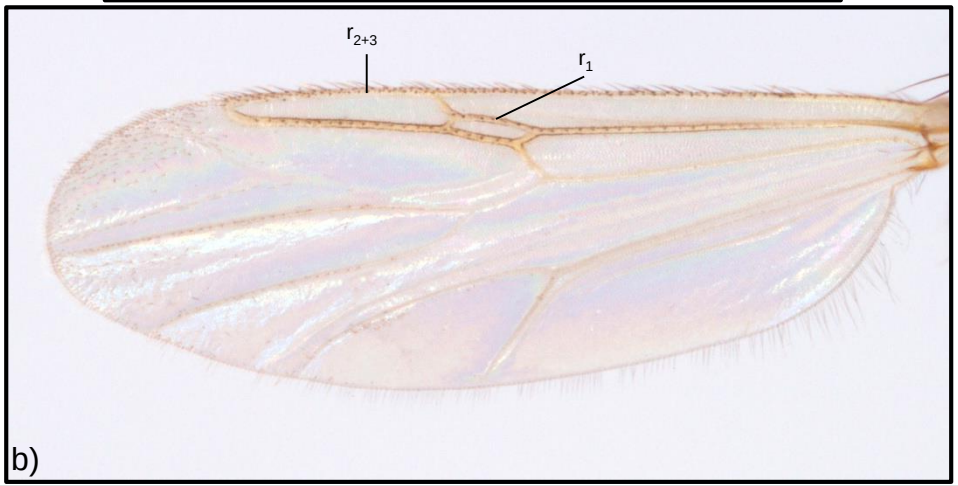


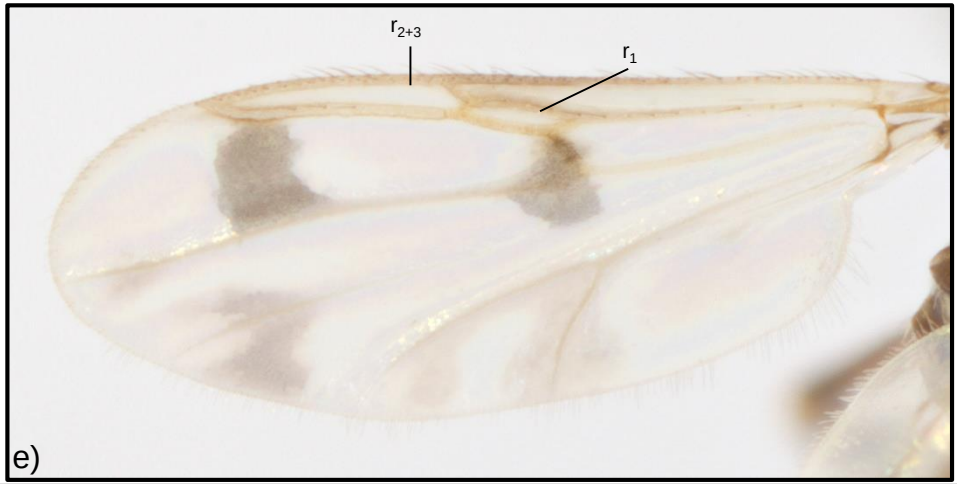


17 (<u>14</u>)	Cells r_1 and r_{2+3} confluent; costal vein, C, long in female, extending past vein R_{4+5} and ending nearly at tip of wing (a); palpus with 4 segments (b).	*Parabezzia*
-	Cells r_1 and r_{2+3} present and separate; C short in female, not extending past vein R_{4+5} and not ending nearly at tip of wing (c); palpus with 5 segments (d).	<u>18</u>

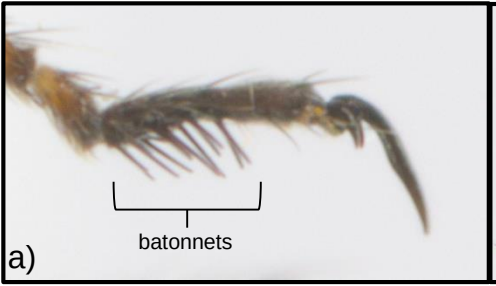


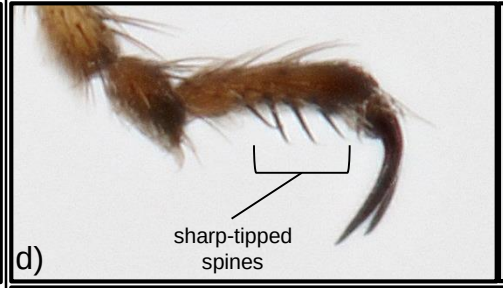


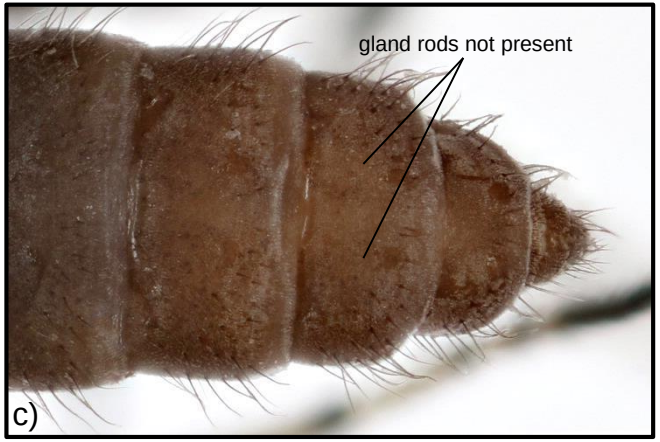


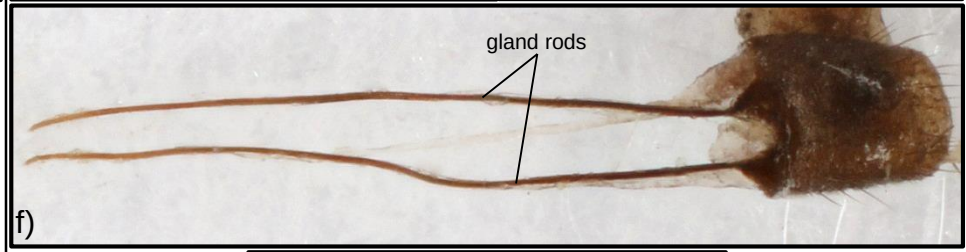


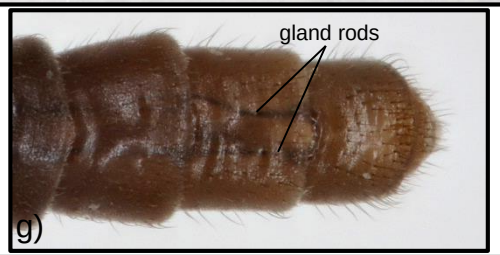
18 (<u>17</u>)	Female claws large and unequal on all legs (a); cell r_{2+3} 2-3 times longer than cell r_1 (b).	*Stilobezzia*
-	Female claws equal on fore and midleg (c); hindleg with one long talon, with or without a short second claw (d); cell r_{2+3}, at most, approximately twice as long as cell r_1 (e).	*Monohelea*



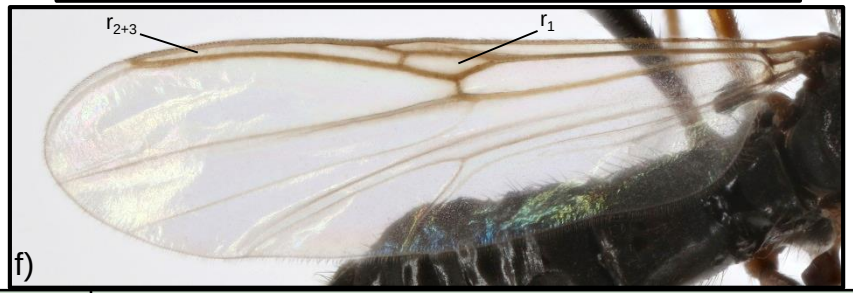




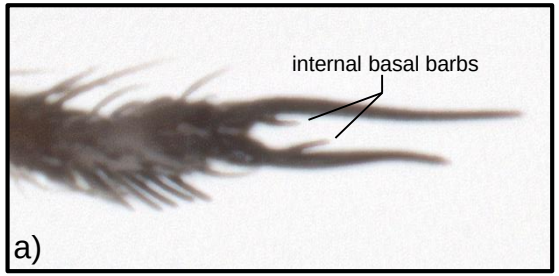


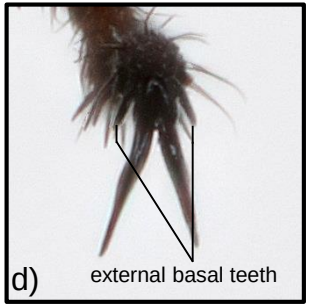


19 (5)	Tarsomere 5 of female armed ventrally with stout black blunt spines (batonnets) (a); sternite 8 of female often with a pair of hair tufts (b); female abdomen without internal sclerotized gland rods (c).	SPHAEROMIINI…….20
-	Tarsomere 5 of female unarmed, **or** armed with slender, sharp-tipped spines (d); sternite 8 of female without pair of hair tufts (e); female abdomen usually with internal sclerotized gland rods, sometimes visible through abdominal tergites (f, g).	26

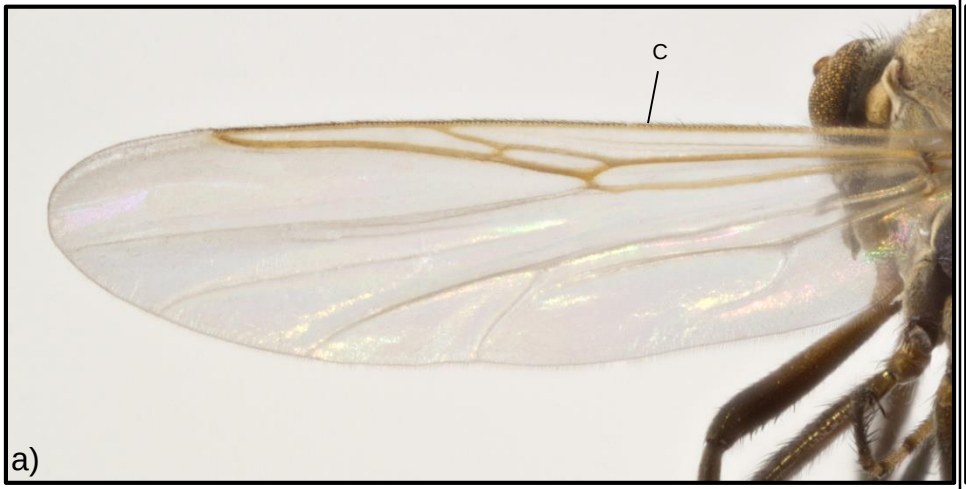


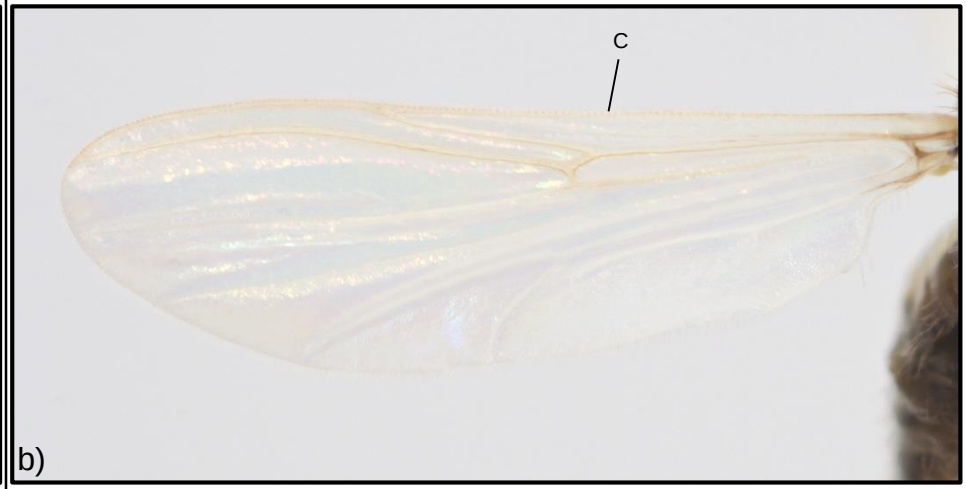
20 (19)	Female tarsal claws equal on foreleg (a) distinctly unequal on midleg (b) and hindleg (c); femora unarmed (d); abdomen petiolate (narrow at base, e); costal vein, C, extending about 0.8 of wing length; cells r_1 and r_{2+3} **usually** both present (f)	*Johannsenomyia*
-	Female tarsal claws equal on all legs (g); femora armed or unarmed (h); abdomen (i) and wing venation (j) various.	21



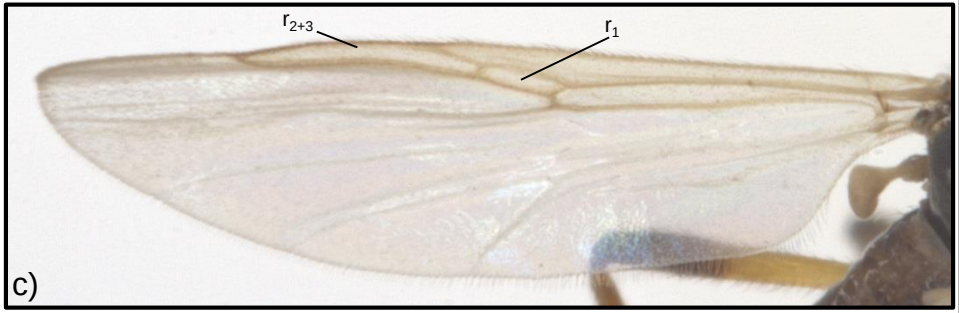


21 (20)	Female claws gently curved distally, and with slender internal basal barb (a); C extending nearly to wing tip (b).	*Sphaeromias*
-	Female claws usually straight or flattened distally, and with blunt external basal tooth (c, d); C various (e).	22

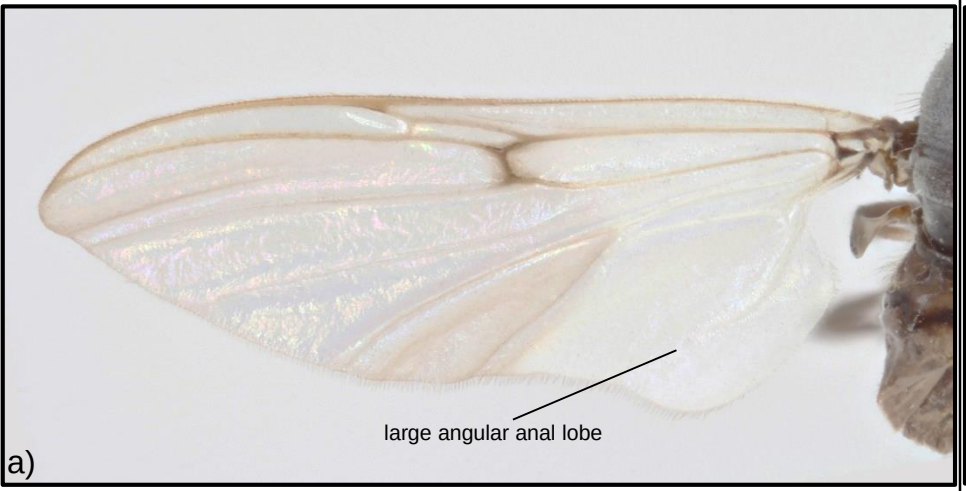




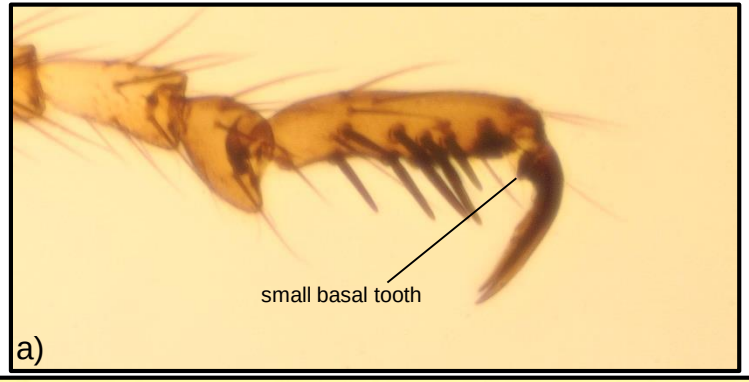
22 (21)	C short, with costal ratio [length of costa / length of wing] approximately 0.8 (a).	23
-	C long, extending nearly to wing tip; costal ratio over 0.87 (b).	24

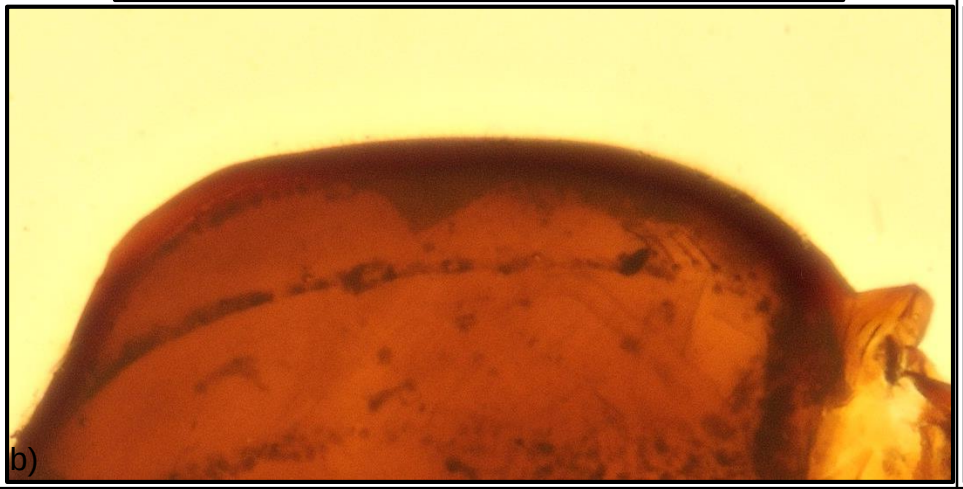


23 (22)	Body slender, with scutum shining yellow to black with little or no pollinosity (a); femora armed or unarmed (b); cells r_1 and r_{2+3} present and separate (c).	*Mallochohelea*
-	Body stouter than *Mallochohelea*, with dull scutum (sometimes with whitish to grayish pollinosity; d); femora armed ventrally (e) and tibiae armed dorsally with numerous sharp spines (f); cell r_{2+3} **sometimes** confluent with cell r_1 (g).	*Nilobezzia*

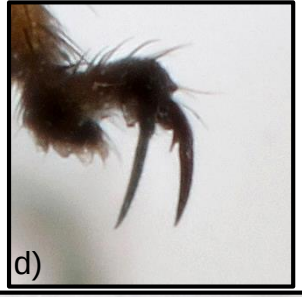


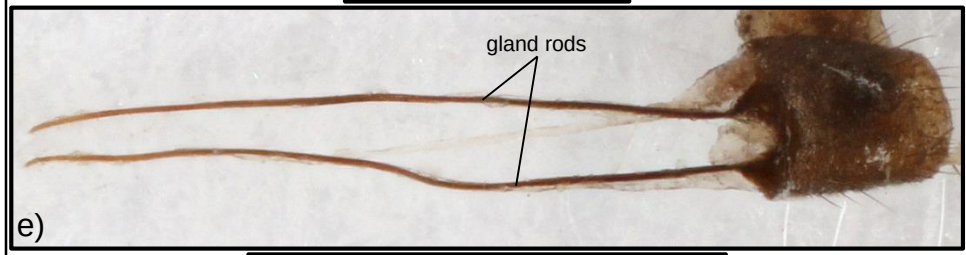
24 (22)	Wing broad, with anal lobe large and angular (a).	*Jenkinshelea*
-	Wing not unusually broad, anal lobe not angularly developed (b).	25

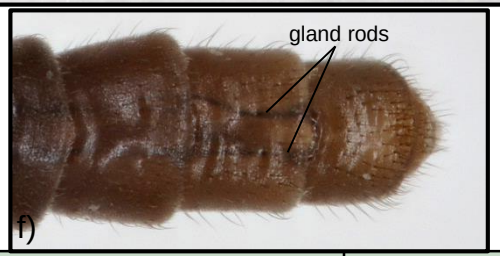




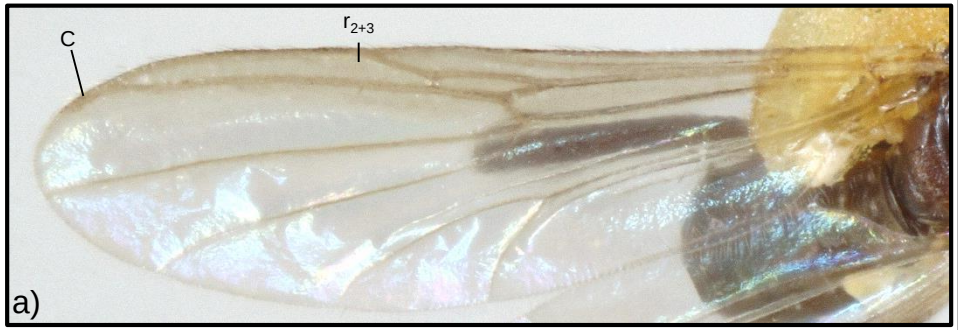
25 (24)	Female claws short, curved, and sharp-pointed, with small external basal tooth (a); scutum without strong, erect bristles (b).	*Macropeza*
-	Female claws long, straight, and somewhat flattened distally, with strong blunt external basal tooth (c); scutum of female with strong, erect bristles (d).	*Probezzia*



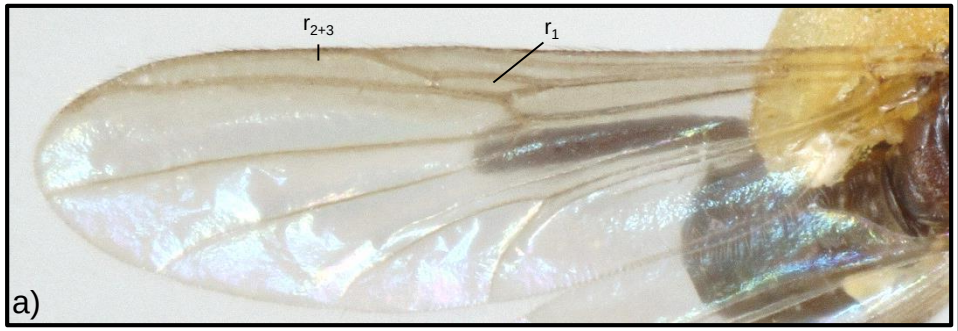


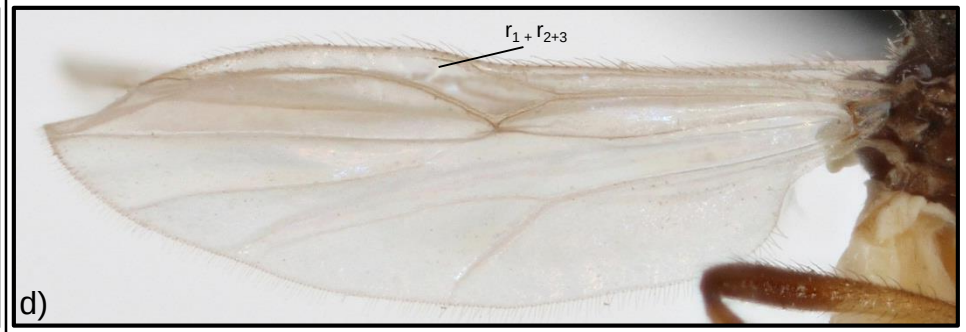


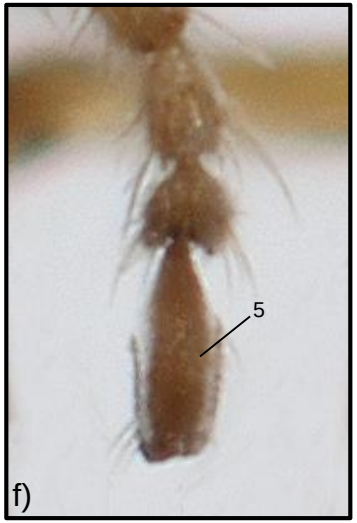
26 (19)	Claws of female unequal, at least on hindleg (a) [**EXCEPT** in one genus, *Neurohelea*, which has equal hindclaws (b)], **OR** hindleg with only a single claw present and with a basal tooth (c); female abdomen without internal sclerotized gland rods (not pictured).	27
-	Claws of female equal on all legs (d); female abdomen usually with internal sclerotized gland rods, sometimes visible through abdominal tergites (e, f).	31



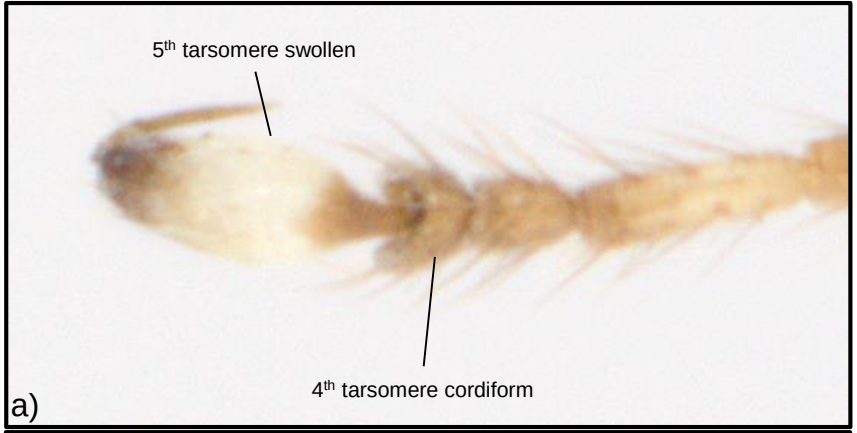
27 (<u>26</u>)	Costal vein, C, extending beyond tip of cell r_{2+3} (a).	<u>28</u>
-	C not extending beyond tip of cell r_{2+3} (b).	<u>29</u>

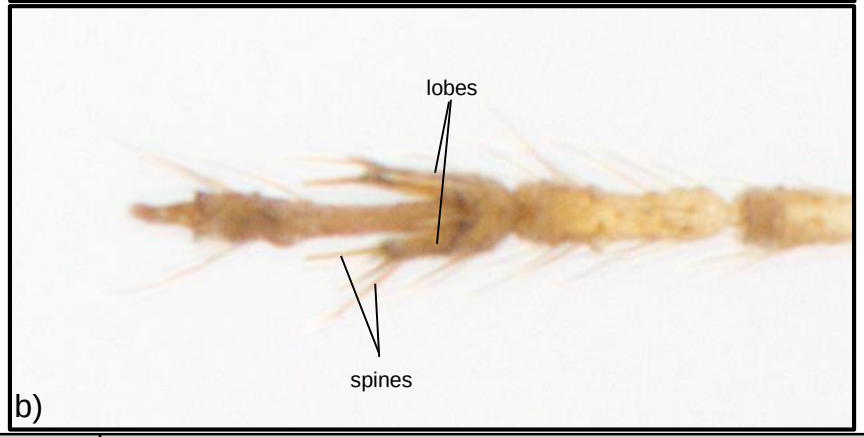


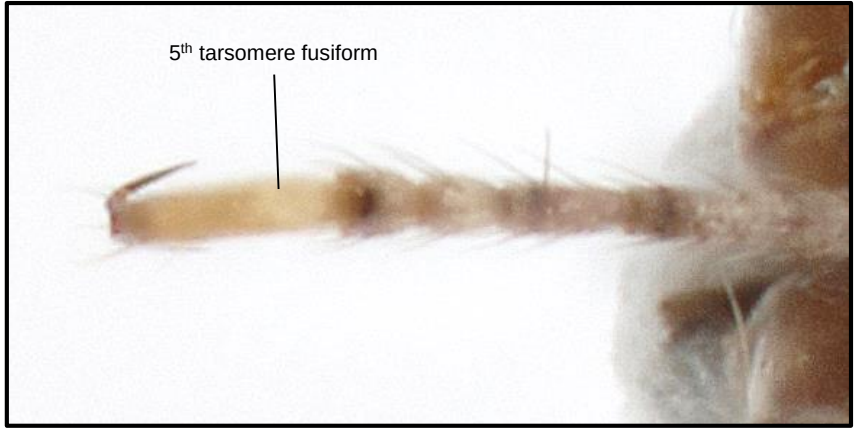




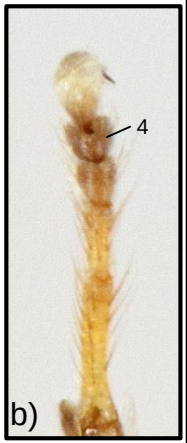
28 (27)	Cells r_1 and cell r_{2+3} present and separate (a); claws equal on all legs (b); tarsomere 5 on foreleg somewhat inflated (c).	*Neurohelea*
-	Cells r_1 and r_{2+3} confluent (d [note: wing somewhat folded in photo]); claws of female equal on foreleg and midleg, but unequal on hindleg (e); tarsomere 5 of foreleg not inflated (f).	*Neurobezzia granulosa*

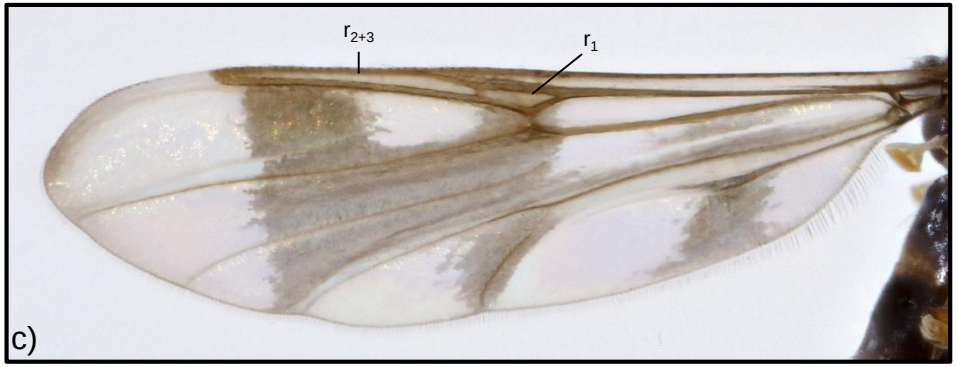


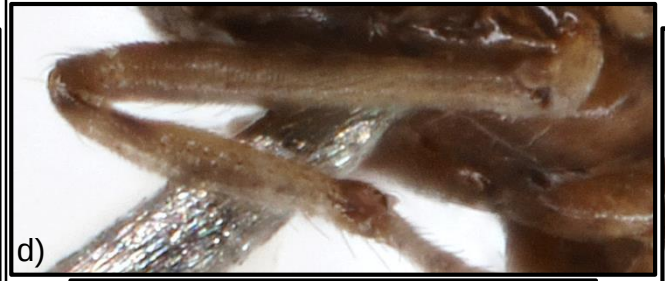


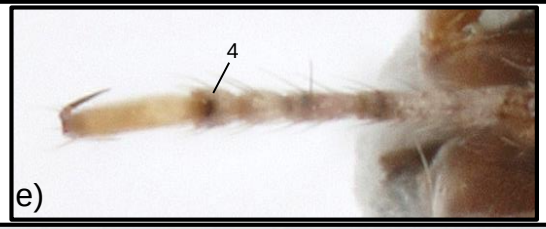


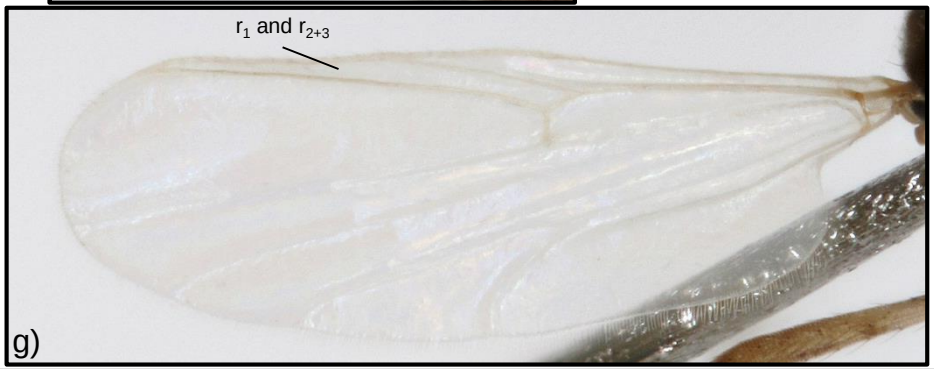
29 (<u>27</u>)	Foreleg with tarsomere 4 cordiform (heart-shaped) and tarsomere 5 greatly swollen (a); mid and hindleg with tarsomere 4 ending in 2 bifid lobes, armed with spines (b).	*Clinohelea*
-	Tarsomere 5 of foreleg somewhat inflated, fusiform (c); tarsomere 4 of mid and hindleg cylindrical or cordiform, but not ending in 2 bifid lobes (d).	<u>30</u>



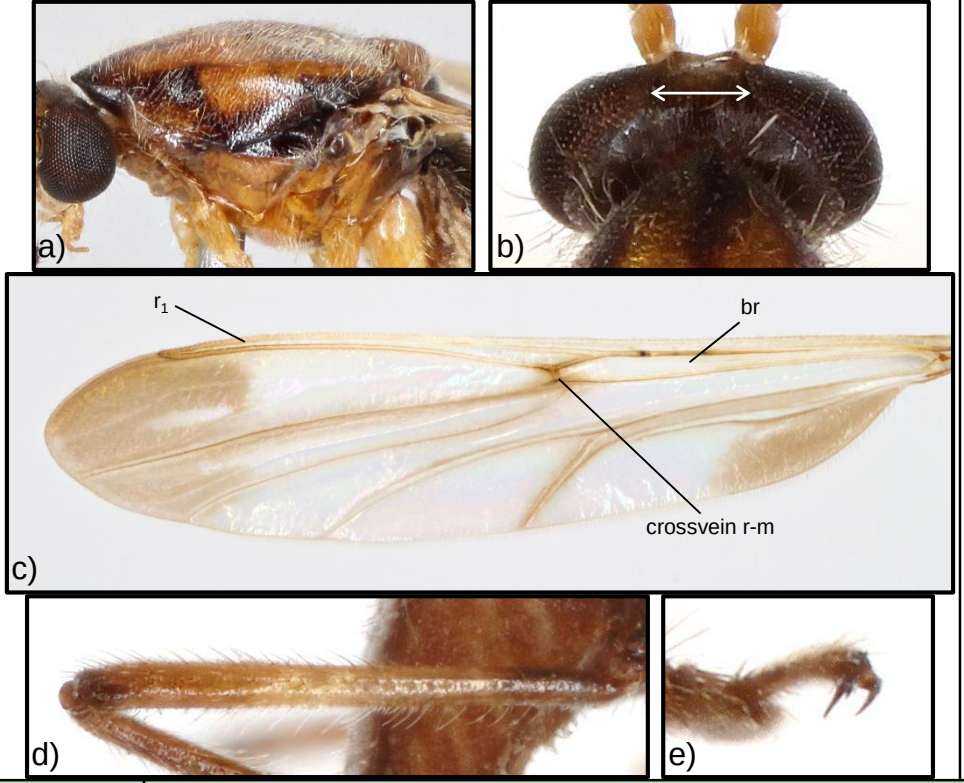


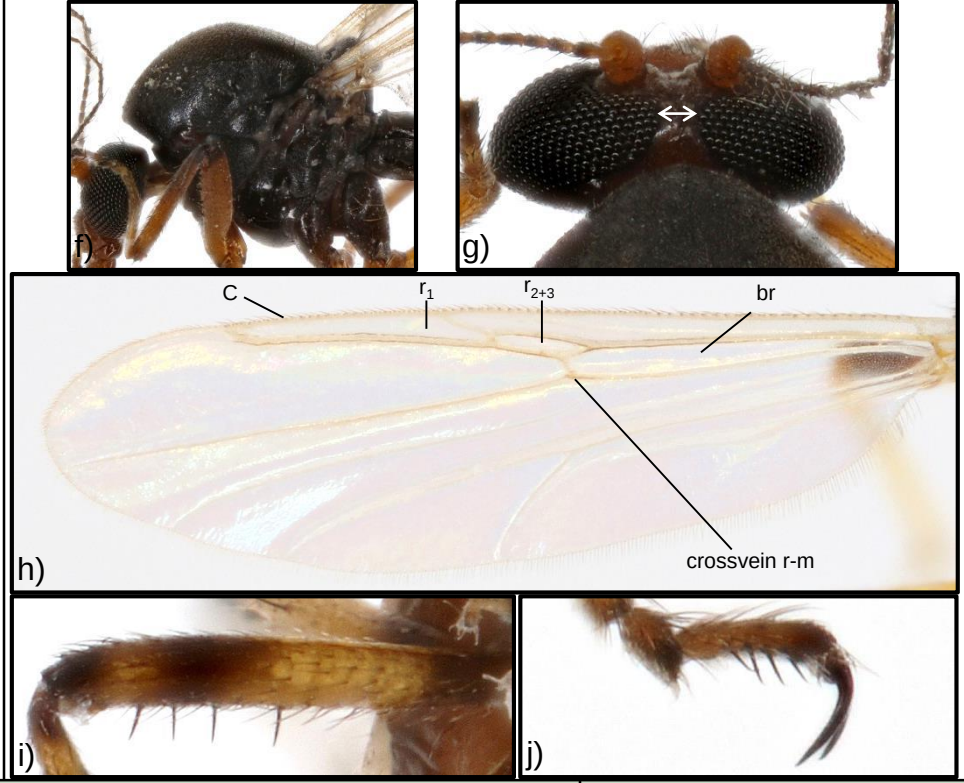




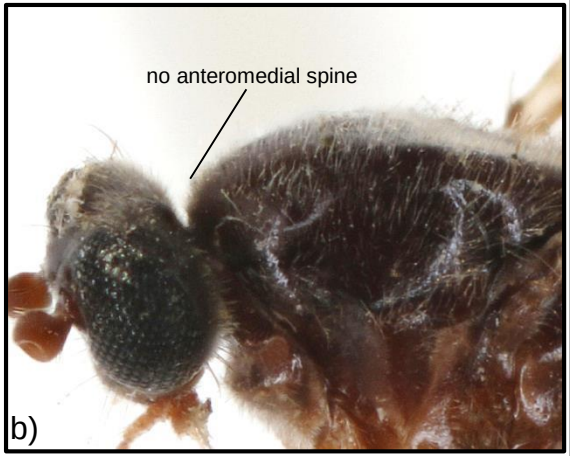


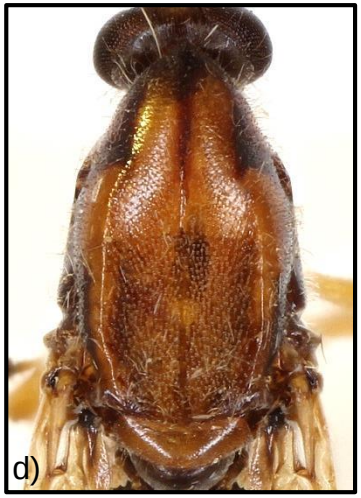
30 (29)	Fore femur swollen, armed ventrally, with tibia arcuate (a); tarsomere 4 cordiform on at least fore- and midleg (b); cells r_1 and r_{2+3} separate or confluent; wing fasciated with darkened patterns (c).	*Heteromyia*
-	Fore femur slender, unarmed, and with tibia not arcuate (d); tarsomere 4 not cordiform (e); body pruinose (with frosted appearance, f); cell r_{2+3} apparently confluent with r_1; wing milky white (g).	*Pellucidomyia wirthi*

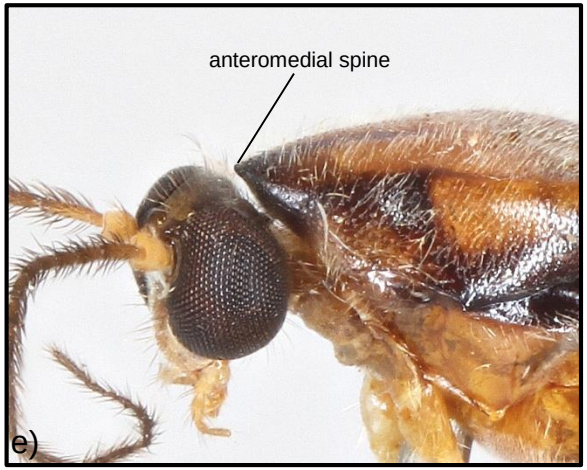


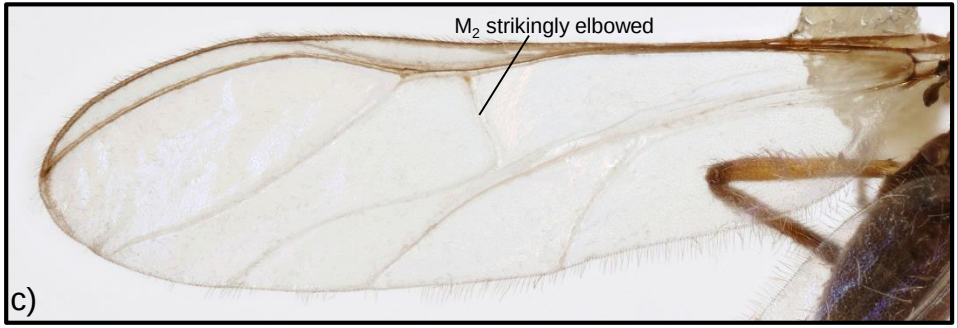


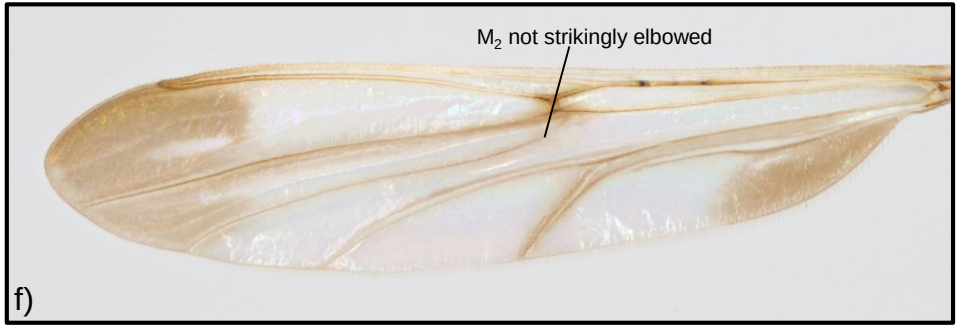
31 (26)	Body slender, dorsoventrally flattened (a); eyes widely separated (b); r_{2+3} absent, r_1 narrow and extending nearly to wing tip in female; crossvein r-m often short; cell br narrow or obliterated (c); femora unarmed (d); claws short (e); legs unusually long and slender (not pictured).	STENOXENINI……..32
-	Body not slender or dorsoventrally flattened (f); eyes narrowly to moderately separated (g); r_{2+3} present **or** absent, but if narrow, C not extended almost to wing tip; r-m longer; cell br well-formed (h); femora often armed (i); female claw often long (j); legs not unusually long (not pictured).	PALPOMYIINI…….33



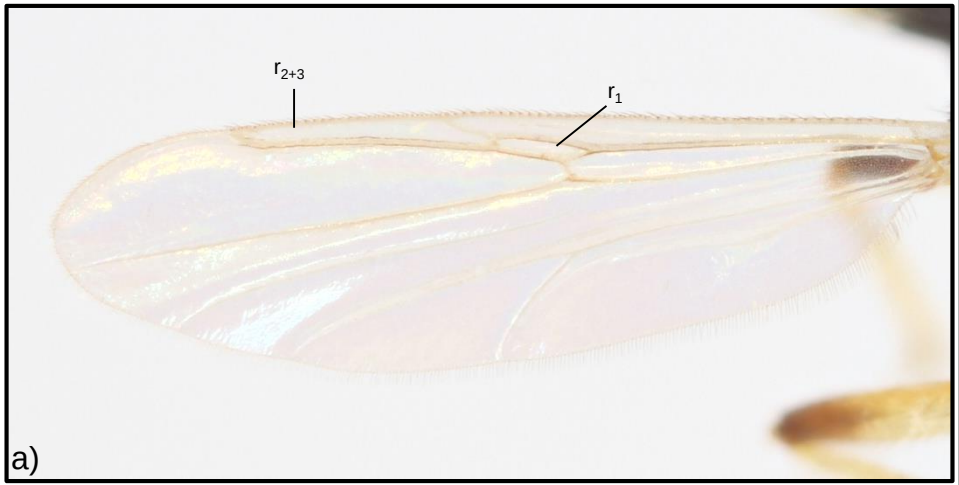








32 (<u>31</u>)	Thorax broadly rounded anteriorly (a), without a median spine (b); female wing with M_2 strikingly elbowed (c); 4 palpal segments (not pictured).	*Stenoxenus johnsoni*
-	Thorax narrowing anteriorly (d), with anteromedial spine (e); female M_2 not strikingly elbowed (f); 5 palpal segments (not pictured).	*Paryphoconus sonorensis*



33 (31)	Cells r_1 and r_{2+3} present and separate (a).	34
-	Cells r_1 and r_{2+3} confluent (b).	35

34 (<u>33</u>)	Hind femur greatly swollen (a); femora unarmed (b); female with pairs of slender spines on tarsomere 5 (c).	*Pachyhelea pachymera*
-	Hind femur not greatly swollen (d); if hind femur moderately swollen, then at least one pair of femora armed ventrally (e); fore femur often swollen (e).	*Palpomyia*

35 (<u>33</u>)	Femora often armed, at least on foreleg (a); female 5th tarsomere without ventral spines (b); costal vein, C, short, with costal ratio ~0.67-0.75 (c).	*Bezzia*
-	Femora unarmed (d); female 5th tarsomere with slender, sharp-tipped ventral spines (e); C longer, with costal ratio higher than 0.75, often ~0.87 (f).	*Phaenobezzia*

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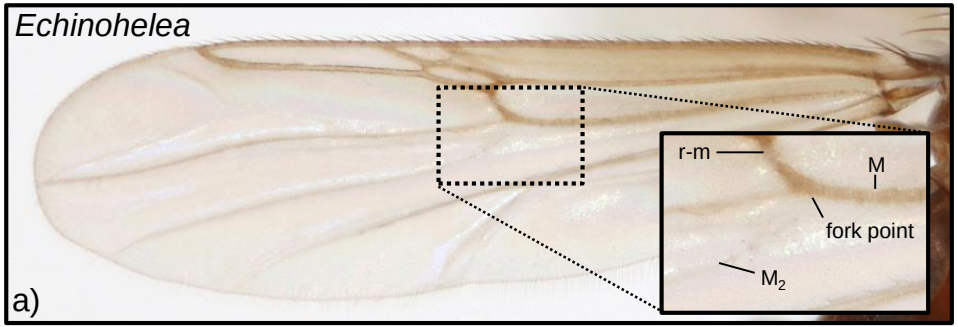
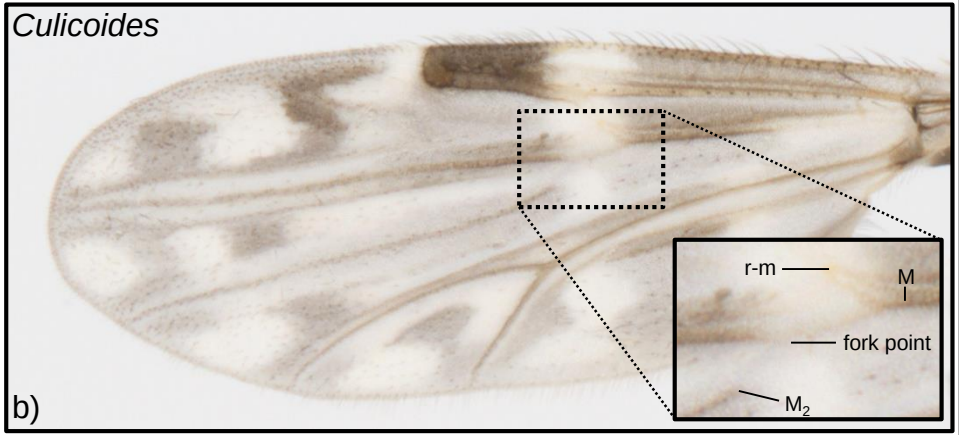
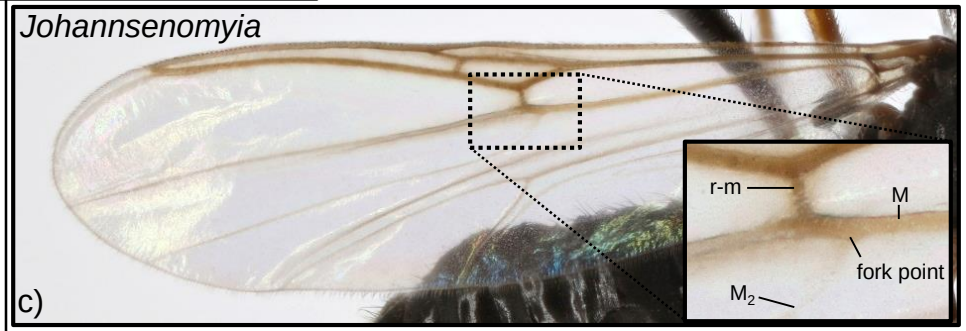
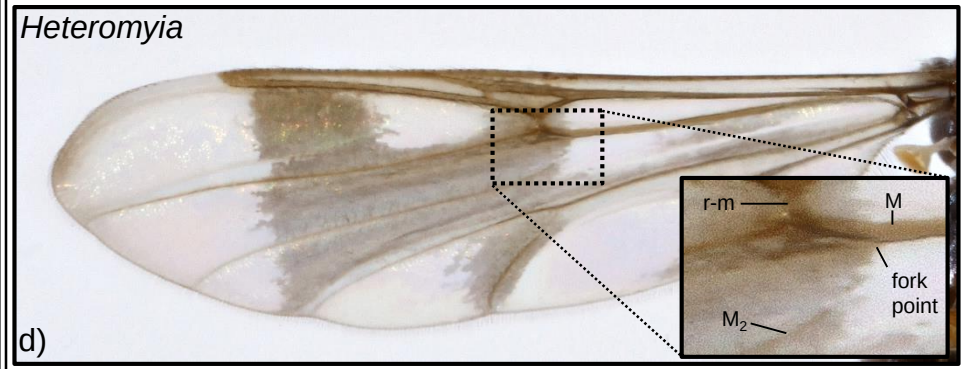








5 (4)	Medial vein, M, forking beyond crossvein r-m, except in spinose-legged genus *Echinohelea* with M forking just at r-m (a); second medial vein, M_2, sometimes obsolete basally (b).	6
-	M forking at or before crossvein r-m, with M_2 **almost** always complete (c, d).	19

Glossary of Terms

Arcuate: Term often used to describe femur or tibia shape, indicates that at least one edge of the structure in question is expanded on one side, giving it an arced appearance.

Batonnet: Stout, blunt, darkened, spines located on the 5th tarsal segment of members of the tribe Sphaeromiini. Batonnets are thickened to almost the distal tip, giving them the appearance of a thick cylinder at lower magnification levels.

Bifid: With two lobes divided by a significant cleft. Often used in reference to a tarsal segment.

Cordiform: Heart-shaped. Often used in reference to a tarsal segment.

Costal ratio: The length of the costal vein divided by the length of the wing, measured from base to distal tip.

Crossvein r-m: A small wing vein that connects the radial vein complex to the medial vein complex. Found in every genus of Ceratopogonidae except *Leptoconops*.

Empodium (pl. empodia): A small filamentous or pad-like structure located between the claws on the 5th tarsal segment.

Fasciate/fasciated: Displays a banded pattern, often resulting in a striped appearance.

Fusiform: Tapered slightly at both ends. Often used in reference to a tarsal segment.

Gland rods: Long, filamentous extensions internally originating from the basal edge of the abdominal tergites. May be visible through the abdominal cuticle, or may require dissection to visualize.

Petiolate: With respect to the abdomen, basal abdominal segments more constricted than those following distally.

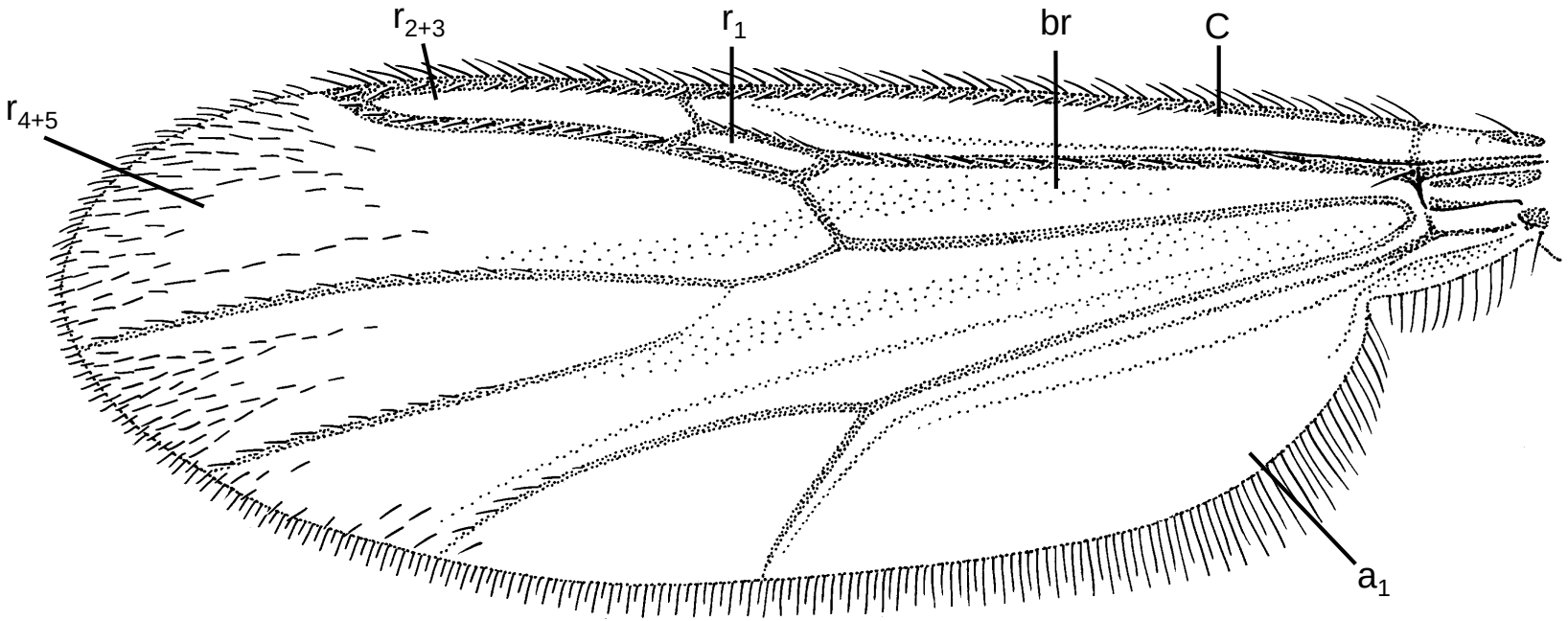
Pollinosity: A structure or segment that is pollinose is considered to have a slightly dusty and non-reflective appearance.

Prescutal pit: Thoracic indentations located near the anterior edge of the prescutum. Prominent in *Culicoides*.

Pruinose: Segment or structure displays a frosted or whitened appearance.

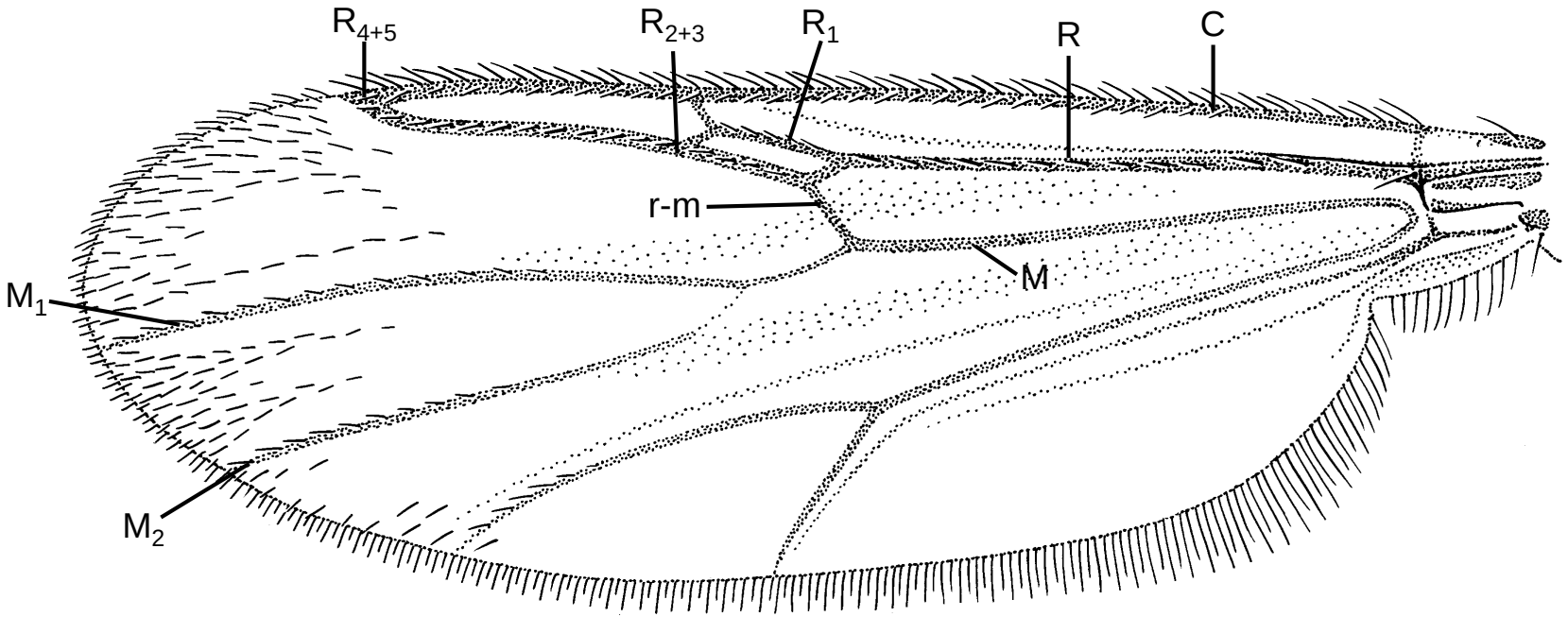
Slender sharp-tipped spines: Found on the 5th tarsal segments of biting midges NOT in tribe Sphaeromiini. Spines are not as stout as batonnets, and appear extremely slender at their distal tip.

Wing Cells Referenced in Key



C: costal cell
br: basal radial cell
r: radial cell complex
a_1: anal cell/anal lobe

Wing Venation Referenced in Key



C: costal vein
R: radial vein complex
M: medial vein complex
r-m: radial-medial crossvein

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Chapter 7: Biting Midge (Diptera: Ceratopogonidae) distribution at three SEMEX facilities located in Guelph, Brockville, and Kemptville, ON in Summer, 2016

7.1 Introduction

Bluetongue virus (BTV) is a debilitating *Orbivirus* disease (family Reoviridae) that can affect a number of ruminants, including important industry livestock such as sheep, cattle, and goats. Originally discovered over a century ago in European livestock that had been transferred to Africa, BTV has since been isolated in a number of other regions, causing numerous severe outbreaks and widespread livestock death. The first recorded instance of BTV in North America was observed affecting a sheep in California in 1952, which had contracted serotype 10 (BTV-10). Since this initial finding, four other serotypes have commonly been isolated across the continent: BTV-2, -11, -13, and -17 (Walton, 2015). There are currently 26 recorded BTV serotypes worldwide (Maan *et al*., 2011); however, many of these have been detected in North America without becoming permanently established. Problematic symptoms of BTV are often observed in goats, deer and sheep, although clinical symptoms only arise in a small fraction of infected animals; many infections are subclinical and go unnoticed. Clinical symptoms that may develop, however, include swelling and haemorrhaging of the nasal and buccal regions, cyanosis of the tongue, esophageal and pharyngeal lesions, excessive salivation, increased body temperature, and haemorrhaging in the hooves. Death may occur when vomit, mucous, and other fluids enter the lungs. Cattle that suffer from infection rarely become symptomatic, and symptoms are much less severe than in animals such as sheep and deer if they develop (Tabachnick, 1996). It has been suggested, however, that cattle may function as reservoir organisms for BTV, allowing it to persist in livestock populations over winter months. Alongside these direct physical effects on livestock animals, BTV can incur a number of indirect costs to farmers as well. Animals infected with BTV require strict quarantine measures for proper

assessment and containment, and confirmed presence of the virus may limit transportation of livestock both into and out of the country.

One of the most significant discoveries regarding Bluetongue virus in the 1970s was the first isolation of BTV in Canada. In 1975, 261 livestock cattle, which had been imported to British Columbia from the United States, were found to be seropositive for BTV and were subsequently destroyed (Thomas *et al*., 1982). Furthermore, 221 cattle that had previously been in contact with the seropositive herd also showed infection when they were tested in 1976. These findings led to establishment of a quarantine zone within British Columbia, which contained a large portion of the Okanagan Valley bordering Washington, U.S.A; any seropositive animals detected within this zone were slaughtered to contain the virus. In the following years until 1979, a country-wide survey for BTV was performed for further monitoring and containment, but only 24 infected cattle were found, a majority of which were isolated within, or in areas adjacent to, the established quarantine zone; two of the infected animals were imported from the U.S.A. Additionally, 15 sheep, 7 goats, and 4 deer within the quarantine zone were found to be seropositive during the survey in 1976. This sharp drop in observed infections led to the conclusion that BTV was no longer present in Canada. For the most part, only occasional instances of BTV antibodies have been observed in livestock near the Okanagan Valley ever since this initial quarantine incident, and nearby farms are under close observation using sentinel livestock herds and other methods (Thomas *et al*., 1982, Clavijo *et al*., 2000).

However, in 2015, a single animal from a cattle farm in the Chatham-Kent region of Ontario was found to have antibodies for BTV when tested at slaughter. Subsequently, two additional cattle from the same farm were found to be seropositive for BTV (OIE, 2015). The cattle had been born in province, and had never been transported off the farm, suggesting that

they contracted BTV from a local source, inducing antibody production. Additionally, research in 2013 yielded insect collections containing the primary vector for BTV in North America, *Culicoides sonorensis* Wirth and Jones, 1957, in several different locations throughout southern Ontario. The species was once again collected in 2014, suggesting that the vector is capable of overwintering in the local climate and could contribute to the spread of BTV if the virus and insect happen to have overlapping distributions (Jewiss-Gaines *et al*., 2017).

The initial finding of BTV-infected cattle in Ontario caused a quarantine to be established, restricting the transportation of livestock animals into and out of the province. This affected numerous livestock producers who were suddenly unable to acquire new animals from out-of-province, or had to maintain animals that were scheduled for transportation elsewhere. Instead, they were required to keep livestock scheduled for transport out of the province under quarantine conditions until the status was lifted. This also included transportation of any material produced by livestock animals, such as bull semen collected for breeding purposes. As such, companies and individuals who depend on livestock farming and production succumbed to a sizeable loss of income as a result of these quarantine measures, with over 1 million dollars in estimated losses for SEMEX Inc. alone; additional financial repercussions still continue, even at present (S. Dunk, director and operations manager at SEMEX headquarters, personal communication, November 13th 2017).

Figure 7.1. *Culicoides sonorensis* (lateral view)

Culicoides sonorensis is a type of biting midge, taxonomically classified in the insect family Ceratopogonidae (Holbrook *et al*., 2000; Figure 7.1). Although this family contains 36 different genera that are native to North America (Downes and Wirth, 1981), only members of the genus *Culicoides* Latreille, 1809 are widely recognized as successful disease vectors due to their habits of biting vertebrate organisms; most other genera either feed on other invertebrates, or only rarely attack vertebrates but don't have the capability to transmit harmful viruses. *Culicoides* species are responsible not only for transmission of BTV, but also other livestock diseases such as Epizootic Hemorrhagic Disease virus (EHDV, also found in North America) and African horse sickness virus (AHSV, found primarily in Africa and the Middle-East). Ceratopogonid flies are notably small in size and are also widely understudied, resulting in a general lack of taxonomists that can identify members of the family. Within *Culicoides* are multiple subgenera, one of which is the subgenus *Monoculicoides*, which contains *C. sonorensis* and its closely-related sister taxa, *Culicoides occidentalis* Wirth and Jones, 1957 and *Culicoides variipennis* Coquillett, 1901 (Holbrook *et al*., 2000). To the untrained eye, these species are incredibly difficult to discern from one another, as they not only share remarkable similarity in

their physical aspects, but also in their genetics, requiring careful study to properly distinguish the species from one another. Since *C. sonorensis* is the only one of these three species that acts as a capable vector for BTV and EHDV in this region, it is important to be able to discern it from its close relatives to gain a proper understanding of its species distribution and take proper precautions to prevent BTV and EHDV infection of livestock animals.

SEMEX Inc. is a biotechnology company with headquarters in Guelph, Ontario, Canada, as well as multiple farms and facilities established throughout Ontario and Québec, Hungary, China, and Brazil. The company focuses on multiple aspects of cattle production, including generation of beef and dairy products, but it also has a strong interest in harvest of bull semen for artificial insemination of cows. The semen is shipped to cattle breeders worldwide to cultivate herds with beneficial genetic qualities. Diseases such as BTV and EHDV pose a significant threat to their objectives, as any indications of cattle infection will incur quarantines on their animals, as well as any transport of material obtained from animals, preventing SEMEX from shipping bull semen to their customers. Additionally, they would not be able to import cattle to their facilities from other locations in order to maintain their herd. As such, the possibility of BTV and EHDV occurring near SEMEX facilities is of great concern and could incur significant losses to the company. For this reason, the author approached SEMEX to propose a collaboration targeted toward assessment of the presence of *Culicoides sonorensis* at three different SEMEX farms located throughout Ontario.

7.2 Materials and Methods

Arthropods were collected at farms that were owned and operated by SEMEX, with headquarters located in Guelph, Ontario, Canada. Farms marked for collection included cattle facilities located in Kemptville and Brockville, Ontario, as well as at the Guelph headquarters. Trapping occurred every Tuesday on a weekly basis, beginning on June 28th and ending on August 31st, 2016.

One John W. Hock CDC model 1312 miniature light trap (Gainesville, FL), powered by a 6-volt battery, was set at each farm (Figure 7.2). Light traps were equipped with ultraviolet light and were baited with carbon dioxide (dry ice) in an insulated container, in which a hole had been drilled to allow CO_2 dispersal. Traps were fitted with fine mesh collection nets, with perforations small enough to restrict arthropods (including Ceratopogonidae) from escaping. The light traps were set within 10 metres of each farm's livestock housing area, where cattle would be located at dusk and dawn; ceratopogonids are known to be most active during these time periods (Downes and Wirth, 1981). Traps were set in the afternoon, left to operate overnight, and retrieved the following morning. Upon retrieval, collection nets were removed from the light traps, closed tightly with a drawstring, and placed in a cooler on ice for transport back to the lab at Brock University. At the laboratory, the collection nets were placed in a -20°C freezer to kill specimens for subsequent sorting, identification, and enumeration, all of which were performed on a chill plate. *Culicoides* specimens of subgenus *Monoculicoides* were further identified to species using a dichotomous key (Holbrook *et al*. 2000), sorted into collection tubes each containing no more than 100 specimens, and re-stored in the freezer. If found, *C. sonorensis* specimens were set aside for RNA extraction and molecular analysis to test for the presence of BTV.

Figure 7.2. Photograph of a CDC model 1312 miniature light trap after setup

7.3 Results

All collection data from the three trapping sites (SEMEX farms located in Guelph, Brockville, and Kemptville) are presented in Tables 7.1 to 7.3.

Table 7.1. Number of biting midges collected (per genus) from collections made at the Guelph SEMEX facility in Summer, 2016.

Guelph	*Forcipomyia*	*Culicoides*	*Atrichopogon*	*Bezzia*	*Alluaudomyia*	*Dasyhelea*
June 28-29	0	0	0	0	0	0
July 5-6	1497	1185	0	0	0	0
July 12-13	2368	330	0	0	0	0
July 19-20	0	0	0	0	0	0
July 26-27	923	32	0	1	0	0
Aug 2-3	4	1	0	0	0	0
Aug 9-10	1187	28	0	0	0	0
Aug 16-17	19	17	0	0	0	0
Aug 23-24	0	0	0	0	0	0
Aug 30-31	0	0	0	0	0	0
TOTAL	5998	1593	0	1	0	0

Table 7.2. Number of biting midges collected (per genus) from collections made at the Brockville SEMEX facility in Summer, 2016.

Brockville	*Forcipomyia*	*Culicoides*	*Atrichopogon*	*Bezzia*	*Alluaudomyia*	*Dasyhelea*
June 28-29	0	0	0	0	0	0
July 5-6	7	0	3	0	0	0
July 12-13	45	2	0	0	0	0
July 19-20	0	0	0	0	0	0
July 26-27	0	0	0	0	0	0
Aug 2-3	10	0	0	0	0	0
Aug 9-10	88	1	0	0	1	0
Aug 16-17	184	0	0	0	0	0
Aug 23-24	443	0	0	0	0	0
Aug 30-31	536	3	0	0	0	0
TOTAL	1313	6	3	0	1	0

Table 7.3. Number of biting midges collected (per genus) from collections made at the Kemptville SEMEX facility in Summer, 2016.

Kemptville	*Forcipomyia*	*Culicoides*	*Atrichopogon*	*Bezzia*	*Alluaudomyia*	*Dasyhelea*
June 28-29	0	0	0	0	0	0
July 5-6	127	0	1	0	0	0
July 12-13	0	0	0	0	0	0
July 19-20	0	0	0	0	0	0
July 26-27	0	0	0	0	0	0
Aug 2-3	1485	0	0	0	0	0
Aug 9-10	0	0	0	0	0	0
Aug 16-17	0	0	0	0	0	0
Aug 23-24	1845	0	0	0	0	0
Aug 30-31	2584	9	0	0	0	16
TOTAL	6041	9	1	0	0	16

7.4 Discussion

Insect collections at all three locations revealed that the biting midge genus *Forcipomyia* Meigen, 1818 was the most prevalent by far, with roughly 6000 specimens collected at both Guelph and Kemptville, and approximately 1300 collected in Brockville over the collection period. This genus is not generally perceived as harmful. Firstly, although certain North American *Forcipomyia* species, such as *F. (Lasiohelea) fairfaxensis* Wirth, 1951, do feed on vertebrate blood, other members of the genus attack invertebrates, such as other larger insects, to gain nutrition (Downes and Wirth, 1981). Secondly, and most importantly, no North American *Forcipomyia* species have been implicated as significant biological vectors of disease. This genus is not a threat to the livestock industry at the moment. However, this is not to suggest that resident *Forcipomyia* species will never become an issue in North America. In some locations, such as Australia, *Forcipomyia* species are under scrutiny as potential vectors for diseases; for

instance, Dougall *et al*. (2011) noted that members of *Forcipomyia* (subgenus *Lasiohelea*) may function as vectors for *Leishmania*. In addition, a particular species of mothfly (Diptera: Psychodidae), *Lutzomyia shannoni* (Dyar, 1929), is a recognized vector of *Leishmania* in North America. If there are locations where *L. shannoni* and *Forcipomyia* co-occur, it is possible that *Leishmania* could be acquired by vertebrate-feeding *Forcipomyia* and further transmitted to additional hosts. It should be noted that Leishmaniasis has been observed in numerous animal species, including some cases in bovine specimens (Lobsiger *et al*., 2010), and as such it could pose a significant threat to SEMEX and the bovine livestock industry as a whole. Therefore, *Forcipomyia* species should not be discounted as potential disease vectors and should likely be monitored in advance.

Overall, the second-most collected genus throughout the season was *Culicoides*, although 99.4% of these (1,593/1,608 total specimens) were collected specifically at the Guelph facility. Examination of these *Culicoides* specimens did not yield any *C. sonorensis*, which function as the disease vector for BTV and other viruses; additionally, no specimens were identified as *C. sonorensis*' closely-related sister species *C. occidentalis* and *C. variipennis*. Instead, nearly all *Culicoides* were taxonomically identified to the *piliferus* group, a subgroup containing approximately 20 species that have not yet been assigned to a specific subgenus, and are not known to contribute to disease spread in North America. As a result, no captured *Culicoides* specimens were subjected to RNA extraction, molecular analysis or disease testing, as they are unable to function as biological vectors for BTV or EHDV.

Members of all other genera that were captured (*Alluaudomyia* Kieffer, 1913; *Atrichopogon* Kieffer, 1906; *Bezzia* Kieffer, 1899; and *Dasyhelea* Kieffer, 1911) pose no threat

to the livestock industry, as they feed on invertebrates and therefore do not function as vectors for disease.

Currently, with respect to these particular SEMEX facilities, it appears that there is no immediate threat of BTV and EHDV infection, as *C. sonorensis* was completely absent from all three sampled locations. However, that is not to say that precautions should not be taken, as species distributions are incredibly dynamic and range expansions can occur very quickly. Harmful insects can be introduced into a new region via simple and common transportation methods. Biting midges are also extremely susceptible to involuntary distribution by aerial wind currents, as their small size makes them struggle to resist high-speed winds (Ducheyne *et al*., 2007). Wind currents are highly suspected to be the method by which African-native *Culicoides* species were introduced into Europe, one species of which became a significant European vector for BTV and contributed to multiple livestock disease outbreaks between 1956 and 1960, as well as numerous other outbreaks since (Ducheyne *et al*., 2007). With the discovery of *C. sonorensis* in various southerly regions of Ontario (Jewiss-Gaines *et al*. 2017), it is likely that the species will expand its range throughout the province via aforementioned distribution methods, and could establish persistent populations near the SEMEX headquarters. It is important to take these aspects into consideration and begin planning for diseases prior to their initial arrival to reduce livestock sickness and fatalities, and prevent the need for quarantine and restricted transportation of animals.

In the near future, it is recommended that SEMEX look into methods by which facilities can further reduce contact between harmful insects and their livestock. Since most vertebrate-feeding insects are generally most active in the early morning and late evening, insect-secured cattle housing facilities would likely be an excellent preventative measure to initial infection.

Insect traps on-site can also reduce the number of biting flies in the area. Finally, making workers aware of the symptoms caused by livestock viruses, so that quick reaction and countermeasures can be put into place upon observing a suspected illness, can be incredibly important for preventing further infection in a herd via insect transmission.

7.5 References

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Chapter 8: Concluding Remarks and Future Directions

8.1 Conclusions

Though most Ceratopogonidae are harmless to humans and mammals, this research concludes that *Culicoides sonorensis* constitutes a significant threat to ungulate animals in Ontario, and perhaps in additional provinces if the species is able to further expand its range. *C. sonorensis'* capability for transmitting viral diseases such as bluetongue virus (BTV) and epizootic hemorrhagic disease virus (EHDV), amongst other potential pathogens, suggests that increased understanding of biting midge ecology would be of great benefit to Canadian agricultural stakeholders. As such, it is important to perform routine collection and assessment of biting midge populations alongside standard insect collection efforts. This would be a simple additional step to help limit the impacts of disease-carrying biting midges on local producers, who may incur significant costs if BTV or EHDV are detected on their premises.

Such routine surveillance could be easily performed by regional public health units, which routinely send travelling employees to set insect traps in order to monitor local mosquito populations. Outfitting CDC Light Traps with a collection vessel composed of finer mesh would allow for capture of biting midges alongside the standard mosquito captures. Traps such as these were used to collect from sheep farms in Chapter 3 of this thesis, and managed to collect hundreds to thousands of biting midges in a single 24-hour collection period. With proper training, entomologists would be able to tally ceratopogonid genera captured in these traps and determine if vector species, such as *C. sonorensis*, are present. Additionally, hunters should be made aware of the impacts of biting midges and educated about the effects and symptoms of BTV and EHDV, as well as other ceratopogonid-vectored animals diseases that may be occurring in nearby regions, provinces, or states. Animals that appear to be displaying such symptoms should then be reported, as well as any dead deer that may be encountered on hunting

excursions, particularly if they are located near bodies of water. Although these practices are often performed by hunters and the general public, reinforcing knowledge and awareness regularly would be an excellent step toward consistently understanding the ranges of biting midges and the viruses they can spread.

In addition to these efforts, Rothamsted traps should be strongly considered for installation and management by the agricultural sector. As mentioned in Chapter 5, Rothamsted traps were used to great effect in Europe for insect monitoring. Though these traps were initially used to collect and interpret the ecological patterns of aphid species, they eventually became integral for understanding *Culicoides* population dynamics during outbreaks of BTV (Fassotte *et al*., 2008). These traps could be used in Canada as well, and may have particularly interesting use with respect to interception of not only Ceratopogonidae, but other insects as well. With respect to biting midges, it may benefit farmers and livestock producers to have Rothamsted traps near their properties with routine inspection by entomologists. During sheep farm trapping with CDC Light Traps as described in Chapter 3, very high numbers of *Culicoides* were captured and *C. sonorensis* was found for the first time in Ontario. These collections occurred only every two weeks, and resulted in a successful interception of a newly introduced viral vector species. The establishment of Rothamsted traps in these types of regions would allow for consistent daily sampling of local biting midge populations, as well as other insects; this would significantly improve the understanding of insect populations and newly-introduced pests into agricultural areas. Upon recognition of a notable species or vector, further surveys using additional collection methods could then be conducted. Additionally, Canada shares an extremely large border with the United States; placing Rothamsted traps along this border would improve the interception of insects exhibiting northward range expansion from the USA. Overall, introduction of

Rothamsted traps in various localities throughout the country could provide a very effective method for understanding the ecology of not only biting midges, but insect fauna in general.

Such trapping practices as listed above are especially important if the trends produced by BTV and EHDV report mapping in Chapter 2 are any indication virus distribution patterns. Throughout the last 60 years, BTV and EHDV have become widespread throughout North America, and it appears that virus incursions into adjacent states or provinces is not uncommon; this is very likely a result of weather patterns and transportation of livestock, or at the very least is facilitated by these distribution methods. Further range expansion of these viruses is likely very possible, particularly in the United States where both BTV and EHDV have been reported within the entire range of latitude. Additional expansion into Canadian provinces, whether by distribution northward from the States or from adjacent provinces is also a threat to consider. As such, sampling for insects such as biting midges, as well as routine livestock scans to assess virus presence or animal seropositivity to viruses, should be performed routinely across the country.

Identification of biting midges can be improved with tools provided by this research. The visual key to the Nearctic genera of biting midges will allow those unfamiliar with ceratopogonid genera to taxonomically identify specimens to genus level, including vector genera such as *Culicoides*. This can then be followed up with additional taxonomic keys, such as Holbrook *et al*. (2000) to determine if a vector species such as *C. sonorensis* has been collected. Additionally, molecular identification of *C. sonorensis* and *Culicoides variipennis* will be improved as a result of the cytochrome oxidase subunit 1 (CO1), internal transcribed spacer 1 (ITS1), and elongation factor 1-alpha (EF1α) sequences deposited in GenBank, as described in Chapter 4. This contribution allows even those without taxonomic expertise to analyze the molecular sequences of collected *Culicoides* to determine if they are *Culicoides sonorensis*. This improved

identification, in addition to expanded surveillance in regions with large-scale agricultural production, would go a long way to ensuring important companies, such as SEMEX (as discussed in Chapter 7) would be better prepared for livestock vectors and the viruses they transmit.

8.2 Future Directions of Study

Perhaps one of the most important subjects that should be investigated with respect to biting midges is the capability for additional species other than *Culicoides sonorensis* to act as vectors for viruses. The first target species for a study such as this should be additional species of *Culicoides* subgenus *Monoculicoides*, the taxon in which *C. sonorensis* and its closest sisters are organized. Additional species in North America belonging to *Monoculicoides* include *C. grandensis*, *C. occidentalis*, *C. riethi*, *C. shemanchuki*, *C. stigma*, and *C. variipennis* (Grogan Jr. and Lysyk, 2015). Due to their close phylogenetic relationship with *C. sonorensis*, it is possible that some of these species may function as biological vectors for BTV, EHDV, or other closely related viruses to at least some degree. Other North American *Culicoides* species should be investigated for vector competence as well, particularly when possible evidence of viruses utilizing additional *Culicoides* species as vectors has been presented in literature, such as *Culicoides insignis* in the southwestern United States (Tanya *et al*., 1992).

In addition, assessment of other bloodfeeding biting midge genera, such as *Forcipomyia* and *Leptoconops* for disease-vectoring capability is another avenue that should be explored more thoroughly. Species of *Forcipomyia*, for example, have been implicated as potential vectors for *Leishmania*, a protozoan trypanosome known to inflict a typically cutaneous infection called

leishmaniasis (Dougall *et al*., 2011). Many *Forcipomyia* specimens were captured in both the Rothamsted trap and CDC Light Traps over the course of this research, suggesting that they are both abundant and widespread in Ontario. If a resident species is capable of spreading a disease, quick range expansion may occur and result in numerous outbreaks, particularly if other insect fauna could also act as vectors or if the disease may be transmitted between non-insect hosts directly. Overall, vertebrate-feeding biting midges should be studied to a moderate degree to understand their ecology, biology, and vector capability to reduce the impact of possible outbreaks in the future.

Climate patterns and their associations with biting midge populations, particularly for *Culicoides* species, would be an excellent topic of study in the future. Since biting midges are often found in the aerial plankton and may be carried moderate distances by wind currents, understanding the generalized patterns of wind movement in Canada would help to assess ceratopogonid distribution patterns. This would be especially important along the Canada/US border, considering that multiple articles and documents have suggested that populations of *C. sonorensis* were carried northward from the states and into the western provinces. Further studies on distances biting midges can be displaced by strong wind would also be useful not only for understanding their distribution in the future, but also for expanding upon how *Culicoides* populations may have been displaced in the past, leading to significant disease outbreaks and introductions of viruses into new regions.

Finally, a taxonomic revision of the genus *Culicoides* would be an excellent step towards improving identification and accurately tracking vector species throughout North America. Borkent and Grogan (2009) comment on the current state of *Culicoides* taxonomy, indicating that there are numerous species lacking official taxonomic placement, species groups that have

yet to be deciphered, and existing dichotomous keys frequently contain unclear and confusing wording. Additionally, there is no single thorough key that allows sufficient identification of *Culicoides* to subgenera or species groups. In addition to this, many *Culicoides* presently require detailed examination of internal or external genitalia for accurate identification. Though this is quite frequent with Dipteran species identification, *Culicoides* require a well-trained eye and a strong microscope due to their small size, particularly for examination of genitalia. These factors in combination make *Culicoides* specimens very hard to identify to species level reliably. Considering that this ceratopogonid genus is of the most economic importance, being able to accurately identify *Culicoides* without such complications would be a great step toward further understanding their impacts and roles in the ecosystem. A photographic key to characters would be ideal, as it would provide a visual representation of these minute characters to make them easier to comprehend; however, the sheer size of these specimens and their discerning traits may make photography incredibly difficult. Perhaps detailed digital drawings, constructed with the aid of photography and generated with imaging software, would be the best option. In addition to this, molecular characters for identified species should be databased when possible to provide gene sequences for molecular identification. This should be done for multiple gene regions per species in order to maximize the number of genetic differences recorded, facilitating more accurate determination.

8.3 References

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