

**CULICIDS ON THE MOVE: A GENETIC CHARACTERIZATION OF TWO
MOSQUITO DISEASE VECTOR SPECIES IN EASTERN INSULAR
NEWFOUNDLAND**

by

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ABSTRACT

Mosquitoes (Diptera: Culicidae) are some of the most significant carriers of human and animal disease causing pathogens on our planet. However in regions where the prevalence of nuisance mosquitoes or diagnosed mosquito-borne illness is low, the motivation for efforts to monitor mosquito populations is often lacking, even though less well-known viral agents, capable of causing significant human or animal diseases, may be active. Recently, populations of two container breeding disease carrying mosquito species have been reported in St. John's, the capital city of the Atlantic Canadian province of Newfoundland and Labrador. The first, *Culex pipiens*, is the primary vector of West Nile virus along the northeast seaboard of North America, while the second, *Aedes japonicus*, is a highly invasive mosquito species reported to play a role in the transmission of West Nile and La Crosse viruses. Here I report the results of two genetic analyses focused on investigating taxonomic identity, genetic diversity, and connectivity of these populations of medically important species. The first chapter utilizes a set of rapid molecular assays to describe the composition of two populations of *Cx. pipiens* on the Island of Newfoundland; that is, populations on the Island are a mix of the behavioural/physiological forms of *Cx. pipiens*. The second chapter uses population genetic techniques to describe the genetic characteristics of a population of *Ae. japonicus* in Newfoundland, as well as how this population may or may not be connected to other population in Canada and Europe. Results of this study indicate a level of genetic diversity within the recently discovered Newfoundland population comparable to populations in other regions and a general lack of structure between Canadian populations and those in Europe.

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Cold and Mosquitoes

Cold and mosquitoes

Those two pests

Come never together

- Ivaluardjuk. In: Rasmussen (1929)

Intellectual culture of the Hudson Bay Eskimos

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1. GENERAL INTRODUCTION

The arthropod family Culicidae – the mosquitoes – contains some of the most prolific animal and human disease vectors on Earth (World Health Organization [WHO] 2014). These cosmopolitan insects are diverse not only in their habitat requirements, but also in their behavior both as immature and adult stages. While the females of many mosquito species require blood meals for egg development, only a small proportion are adapted to the blood of humans and many species don't require a blood meal at all. Though the ecological roles played by all mosquito species are important in understanding this diverse group of organisms, it is the human blood-feeding species that are of the highest concern for human health. While many insects can act as carriers of a diverse range of pathogens, it is the activity of these vector mosquitoes that holds the highest impact on human health, even when considering malaria alone (Lemon *et al.* 2008). Human driven changes affecting insect habitats not only disrupt existing community structure, but may also serve as a driving force promoting the spread of some of the most common carriers of zoonotic pathogens (Vora 2008). While many of the most well studied disease carrying mosquitoes exist in tropical regions (e.g. malaria carrying *Anopheles* mosquitoes), the ability for vector mosquitoes to establish populations in novel regions does represent a significant risk to human health worldwide. However in regions where mosquito prevalence or diagnosed mosquito-borne illness is low, the motivation for efforts to monitor mosquito populations is often lacking, even though less well-known viral agents may be active. Recently, populations of two container breeding disease carrying mosquito species have been reported in St. John's, the capital city of the Atlantic Canadian province of Newfoundland and Labrador. The first, *Cx. pipiens*, was initially collected in communities in western Newfoundland

during a West Nile virus survey in 2004. However, at the time it was suggested that this species could not survive in St. John's due to its intolerance of cold climatic conditions and a lack of suitable habitat (Hustins, 2006). Notably, this species is the primary enzootic vector of West Nile virus along the northeastern seaboard of North America (Turell et al. 2005). The second species, *Aedes japonicus* (Theobald), is an invasive mosquito species native to Japan and Korea that has undergone a rapid and widespread invasion of North America and Europe (Tanaka et al. 1979; see Kaufman and Fonseca 2014). Since the onset of its incursion into North America, *Ae. japonicus* has tested positive for a number of important human pathogens including West Nile virus and La Crosse virus (Scott 2003; Harris et al 2015).

For reasons including the identification of routes of entry into new regions, or to understand the ability for these species to expand their range along the fringes, it is important to consider populations of species as they migrate throughout novel environments. The major overarching theme of this thesis is to investigate novel populations of these two mosquito species on the Island of Newfoundland (hereafter the Island or the Island of NF); specifically, using genetic techniques to investigate taxonomic identity, genetic diversity, and population connectivity. I address this theme in two parts; the first surrounding the *Cx. pipiens* species complex in which I use PCR based rapid identification techniques to both reaffirm historical collections in western Newfoundland, and to characterize both historic and recently established populations in a taxonomically detailed way. Previous work in the province on this species complex has identified that members of this complex exist in Newfoundland; however no further work was done to identify which complex members may be present, an important factor when considering disease risk. This chapter has been published in the Journal of Medical Entomology and is formatted as such. The second chapter covers a population genetics study of a recently

discovered population of *Ae. japonicus* on the Island. Populations of *Ae. japonicus* in Newfoundland, Nova Scotia, Ontario, and Europe were analyzed using a combination of nuclear (microsatellite) and mitochondrial loci. Genetic characteristics of all populations are presented and connectivity between Newfoundland and other populations are discussed. This chapter has not yet been published but is formatted to match the first for consistency.

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2. THE ARRIVAL OF THE NORTHERN HOUSE MOSQUITO *CULEX* *PIPIENS* (DIPTERA: CULICIDAE) ON NEWFOUNDLAND'S AVALON PENINSULA*

2.1 ABSTRACT

Culex pipiens L., the northern house mosquito, is the primary vector of West Nile virus to humans along the east coast of North America and thus the focus of much study. This species is an urban container-breeding mosquito whose close contact with humans and flexibility in host choice has led to its classification as a "bridge vector"; that is, it is thought to move zoonotic diseases to humans from vertebrate reservoirs. While this vector species is generally very well studied, populations along the fringes of its range are less well known. Here we report, using morphological and genetic techniques, the existence of two locations where *Cx. pipiens* exists in Newfoundland in both expected and unexpected sites based on projected habitat suitability on the island. In addition, we characterize these populations in terms of their composition between the two known behavioral/physiological forms of *Cx. pipiens*.

2.2 INTRODUCTION

Members of the arthropod family Culicidae - the mosquitoes - are significant vectors of human disease-causing pathogens, leading to over 1 million human deaths annually (World Health Organization [WHO] 2014). While the impact of mosquito-vectored zoonotic arboviruses on human health in Canada is relatively low compared to more southern regions of North America, endemic and introduced pathogens such as snowshoe hare virus, Jamestown Canyon virus and St. Louis encephalitis virus are present (Hongoh et al. 2009). Of these mosquito vectored zoonotic arboviruses West Nile virus (WNV) has garnered a lot of attention in Canada due to its relatively recent introduction and its apparent high incidence rate and morbidity compared to other arboviral encephalitides (Pepperell et al. 2003), albeit this might be due to underreporting of other arboviral infections (Drebot 2015). Since the first confirmed human WNV infection in Canada in 2002, 5,230 human cases have been confirmed with the majority of cases being reported in Eastern Canada (Health Canada 2016). Upon its introduction to Canada, efforts to monitor this disease focused on humans, susceptible hosts such as crows and horses, and on the arthropod vectors (Health Canada 2016). However, current WNV-monitoring efforts in Canada focus primarily on the vectors – mainly *Culex tarsalis* in the western provinces, and *Culex pipiens* L. – the focus of this manuscript - in the east (Turell et al. 2005, Drebot et al. 2003).

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Culex pipiens L. is a member of the *Culex pipiens* complex. Although there are several species in the complex, only two have a worldwide distribution: the nominal species *Cx. pipiens*, and *Culex quinquefasciatus* Say (Farajollahi et al 2011). *Culex pipiens* exists as two behavioural and physiological variants, *Cx. pipiens* form *pipiens* L. and *Cx. pipiens* f. *molestus* Forskal (Harbach et al. 1984). In contrast to *Cx. pipiens* f. *molestus*, *Cx. pipiens* f. *pipiens* always requires a blood meal to produce the first egg raft and does not mate in confined spaces. Importantly, females of this form are capable of entering diapause in the fall retreating to humid caves or human structures where they survive cold winters (Fonseca et al. 2004, Farajollahi et al. 2011). Studies of host-preference in these forms have shown that while *Cx. pipiens* f. *pipiens* feeds primarily on birds, *Cx. pipiens* f. *molestus* feeds readily on both avian and mammalian hosts (Ciota et al. 2013). Recent analyses have shown a high degree of hybridization between these forms in North American populations relative to European *Cx. pipiens* f. *pipiens* populations (Fonseca et al. 2004). Additionally, hybridization between these forms has been shown to negatively impact host specificity and increase vector capacity to transmit WNV to humans (Ciota et al. 2013). These hybrids can therefore act as bridge vectors transmitting zoonotic agents between birds and mammalian hosts, particularly humans (Hamer et al. 2008, Huang et al. 2009, Osório et al. 2012, Fritz et al. 2015).

Within North America, the distribution of the *Culex pipiens* complex is well documented; specifically, the two worldwide species are present, and both are known vectors of significant arboviral pathogens. Both species are associated with humans and thrive in cities and suburbs, but while *Cx. pipiens* is found in northerly temperate areas, *Cx. quinquefasciatus* inhabits more southern, sub-tropical to tropical regions (Farajollahi et al. 2011). Additionally, an extensive hybrid zone exists where the ranges of these species overlap (Huang et al. 2011; see Fig. 2.1 for

species distribution and hybrid zones). In Canada, *Cx. pipiens* has been shown to inhabit regions along the southern border, extending eastward into the Atlantic Provinces, but it is unclear how each of the forms of *Cx. pipiens* are present in Canada.

Mosquito collection efforts in the province of Newfoundland (NF) have been sporadic with most collection efforts taking place in the 1970s and early 1980s (Pickavance et al. 1970, Wood et al. 1979, Nielsen and Mokry 1982; Appendix I, II). However, amidst the heightened awareness of mosquito borne diseases after the introduction of WNV to Canada, a renewed collection effort in NL took place in 2004 - 05. During this period a mosquito collection regime was duplicated in two communities on the island of NF: in Deer Lake (49°10'28"N and 57°25'37"W) on the west coast of NF, and approximately 400 km to the east in the province's capital, St. John's (47°34'3"N and 52°42'26"W; Hustins 2006). Deer Lake lies within the rugged and heavily forested Western NF ecoregion where summers are much warmer and longer compared to the maritime barrens ecoregion where the coastal city of St. John's is located (Damman 1983). In addition to these focal sampling locations, 11 parks scattered across the Island were monitored using a reduced sampling effort (Hustins 2006). Over the collection period, the first collection of *Cx. pipiens* complex members on the Island was made at three sites on the west coast of the Island (36 specimens over two years, Hustins 2006), though no evidence of this species was found in St. John's. Specimens from this collection from Deer Lake (Fig. 2.1 – B) were identified using standard taxonomic keys (Darsie and Ward 2005, Thielman and Hunter 2007) and a subset of these specimens were examined using a rapid assay designed to separate members of the *Culex pipiens* complex from other morphologically similar species (e.g. *Cx. restuans*) (Hustins 2006, Crabtree et al. 1995). However, while this assay can identify members of the *Cx. pipiens* complex, it cannot identify the different species or forms within the

complex. Of note, the results of this study identified populations of *Culex pipiens* complex on the west coast of the Island only (Hustins 2006).

Hongoh and colleagues (2012) predicted that under climate change habitat in eastern NF would become suitable for *Cx. pipiens* by 2011 while western portions of the Island would become suitable much later (Fig. 2.1). Supporting this prediction, a single mosquito specimen resembling *Cx. pipiens* was collected inside a home on the Avalon Peninsula in 2012 (Bassett 2014). This mosquito was later identified as likely being *Cx. pipiens* using standard morphological keys and COI DNA sequence (Genbank accession no. KF761601, Bassett 2014). While these methods do not provide a definitive identification of the members of the *Culex pipiens* complex (Laurito et al. 2013), this find motivated our survey in 2013 predicated at determining the range of *Cx. pipiens s.l.* within the city.

However, as mentioned, identification of many *Culex* species (including *pipiens* spp., *restuans*, *salinarius*, *torrentium*, etc.) based on physical characteristics is often problematic due to their morphological similarity and the fact that critical characters are easily destroyed during collection (Harrington and Poulson 2008, Laurito et al. 2013). Importantly, we wanted to be certain of our identification since our findings had the potential to represent the first report of significant taxa to the region. Fortunately, a number of polymerase chain reaction (PCR)-based rapid identification techniques have been established in recent years in order to streamline the genetic identification of similar *Culex* species in North America (Crabtree et al. 1995, Smith and Fonseca 2004, Bahnck and Fonseca 2006). In summary, the objectives of the current study were to confirm prior reports of the presence of *Cx. pipiens s.l.* in western NF, to provide a more definitive identification of all *Cx. pipiens s.l.* collections in NF, and further examine the range of these populations on the Island.

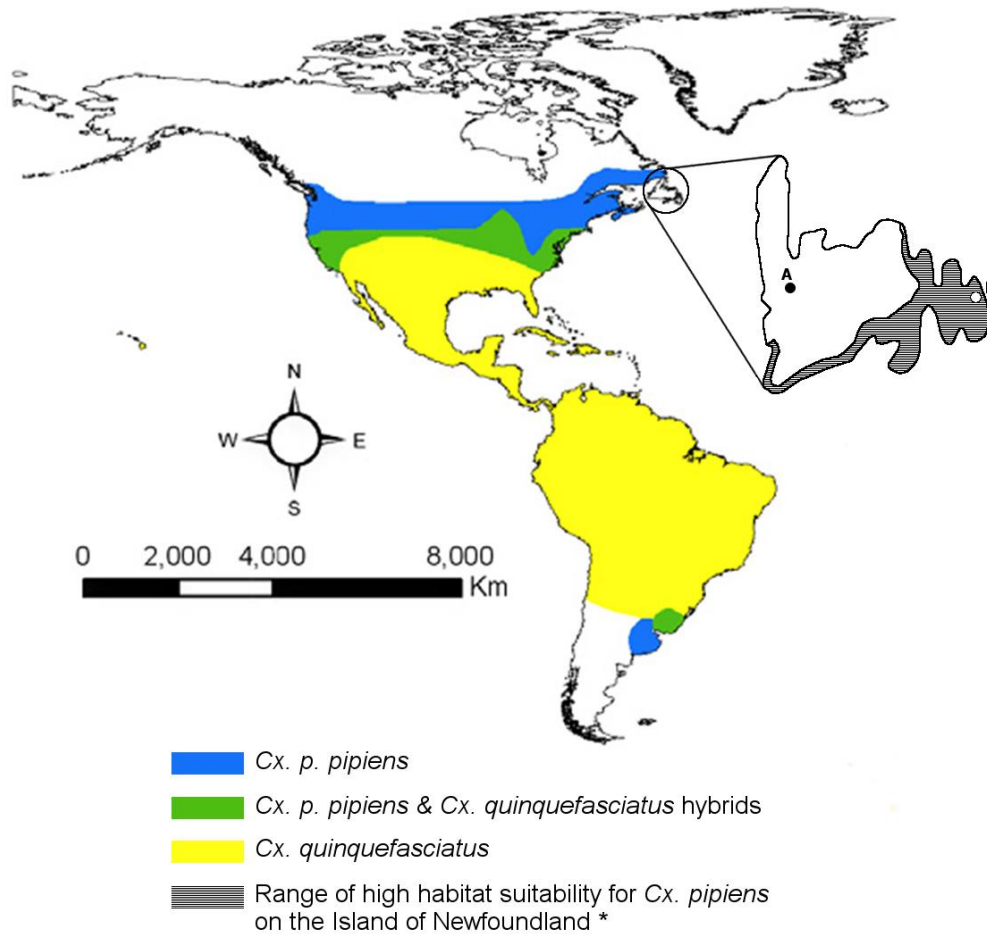


Figure 2-1: Map of North and South America showing the known range of all members of the *Culex pipiens* complex present in this region. The Island of Newfoundland is emphasized showing the projected habitat suitability for *Cx. pipiens* between 2011 - 2040 (Hongoh et al. 2012). Collection locations for the present study are indicated by A (Deer Lake; Hustins 2006) and B (St. John's, 2012-13). Reproduced with permission and modified from Farajollahi et al. (2011).

2.3 MATERIALS AND METHODS

2.3.1 Sources of *Culex* specimens

We obtained mosquitoes from two primary sources. First, pinned and identified specimens collected by Hustins (2006) and stored at Memorial University of Newfoundland were utilized. Thirty-six (36) specimens identified either as *Cx. pipiens*/*Cx. restuans* or *Cx. pipiens s.l.* were collected from the west coast of the Island (Deer Lake region) in 2004 – 05 of which 10 were retained in the Memorial University collection. The specimens collected in 2004 were identified as *Cx. pipiens*/*Cx. restuans* using standard morphological keys, and genetically as members of the *Culex pipiens* complex using the Crabtree assay (Crabtree et al 1995) while the specimens collected in 2005 were identified morphologically and assumed to be *Cx. pipiens s.l.* (no genetic analysis). Second, during the early fall of 2012 and summer of 2013 we collected specimens on the east coast of the Island, within the city of St. John's. A single *Culex pipiens*/*Cx. restuans* specimen was collected serendipitously in 2012 using a hand-held mouth aspirator. To investigate further, in the early spring of 2013, we targeted this species in St. John's by using artificial oviposition traps constructed from 11.4 L translucent white plastic buckets filled with a water based infusion of typical yard waste to a depth of 5 cm (~1.7 L) (see Fielden et al. 2015; Appendix III). We placed twenty-seven traps in residential sites in east St. John's covering an area of approximately 4 km². We inspected the traps weekly for the presence of immature stages of mosquitoes. If present, samples were taken to Memorial University and reared to adults in breeders (BioQuip, Rancho Dominguez, CA, Item #1425). The larvae were fed a diet of fishmeal ground to a fine powder and upon emergence the adult mosquitoes were continuously given access to cotton balls soaked in a 10% sucrose solution in water. Adult

specimens were killed by freezing them at -20°C for a minimum of 24 hours prior to pinning. Positive controls from colonies of *Cx. pipiens s.l.* and *Cx. quinquefasciatus*, and one field collected *Culex restuans* (Oakville, ON, 2003) were provided by Rutgers University (NJ, USA), the Florida Medical Entomology Laboratory (Vero Beach, FL), and Brock University, respectively. All specimens were allowed to air dry prior to identification and processing. NJ and FL specimens were air dried and stored at -80 °C while all other specimens were pinned and stored at room temperature. Vouchers are stored at Memorial University in room SN4113.

2.3.2 Morphological Identification

Hustins' specimens (Hustins 2006) were identified anew using standard taxonomic keys (Darsie and Ward 2005, Thielman and Hunter 2007), as were all specimens we collected in St. John's. Identifications of the St. John's specimens were corroborated by the National Identification Service program (determined by B. Sinclair) offered by Agriculture and Agri-Food Canada (additionally, one of these specimens was retained for the national collection) and by Dr. Roxanne Connelly at the Florida Medical Entomology Laboratory (Vero Beach, Florida). However, in regions where *Cx. pipiens* spp. overlap with morphologically similar species or when specimens have become damaged during collection, it has become standard and advisable to perform molecular analyses in addition to morphological keys (Apperson et al. 2002, Harrington and Poulson 2008, Andreadis et al. 2005, Johnson et al. 2015).

2.3.3 DNA Extraction and PCR Protocols

We extracted DNA from 19 specimens: a single *Culex pipiens/Cx. restuans* specimen collected in St. John's in 2012, eight collected in 2013, and 10 from the west coast collections (2004 – 4, 2005 – 6). Additionally, DNA from two specimens of each positive control group was

also extracted. Pinned adult specimens were photographed dorsally and laterally using a Leica DFC420 digital microscope camera mounted on a Leica MZ95 compound microscope before being individually macerated for DNA extraction using 100 µl pipette tips that were formed into a pestle by briefly melting the ends of the pipette tips in a gas flame. DNA extractions were performed using the Qiagen DNeasy Blood and Tissue kit and provided protocol (DNeasy Tissue Kit, Qiagen, Valencia, CA), and completed extractions were stored at -20 °C. DNA concentration and overall quality of the extracts were evaluated using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE) and samples were selected for further analysis based on overall quality of DNA extract.

Identification of *Culex* specimens to species was performed in three steps using diagnostic PCRs that exploit sequence polymorphisms in various sections of the mosquito genome. The first assay was used to differentiate between *Culex pipiens* complex mosquitoes and select morphologically similar species (*Cx. salinarius* and *Cx. restuans*) (Crabtree et al. 1995); the second was used to distinguish between *Culex pipiens*, the temperate species and *Cx. quinquefasciatus*, the tropical species (Smith and Fonseca 2004); and the third was used to separate the two behavioral/physiological forms of *Cx. pipiens*, f. *pipiens* and f. *molestus* (Bahnck and Fonseca 2006). The first PCR was carried out in 15 µL volumes containing 1X GoTaq Colourless Master Mix (Promega, Madison, WI), 0.4 µM each primer, and 1 µL DNA extract. The ACE-locus PCR was also performed in 15 µL volumes, contained 1X GoTaq Colourless Master Mix, 1 µL DNA extract, and primer concentrations followed those reported in Smith and Fonseca (2004). Finally, the third PCR was performed in 20 µL volumes containing 1X DreamTaq Green Master Mix (Thermo Fisher Scientific, Waltham, MA), 1 µL DNA extract, and primer concentrations reported in Bahnck and Fonseca (2006). The PCR conditions were

identical to those reported in the original publications (Crabtree et al. 1995, Smith and Fonseca 2004, Bahnck and Fonseca 2006). Fragment sizes of all PCR products were visualized with UV light on 1.5% or 2% agarose gels stained with RedSafe Nucleic Acid Staining Solution (iNtRON Biotechnology Inc., Sungnam-Si, Gyunggi-Do) and sized by comparison to a 100 bp ladder (New England BioLabs Inc., Whitby, Ontario; Froggabio, Toronto, Ontario).

In cases where samples amplified using the Crabtree et al. (1995) assay failed the ACE-locus test, likely due to DNA degradation, an alternative analysis was used to aid in specimen identification. This was done because poor DNA quality can lead to poor or no amplification (especially regarding nuclear DNA; i.e. the ACE-locus), and the possibility that these specimens might be of other closely related species could not be ignored. To investigate this possibility, we amplified and sequenced a portion of the mitochondrial ND4 locus. PCRs were conducted in a Veriti thermal cycler (ABI, Fisher, Thermo Fisher Scientific, Waltham, MA) in 20 µl volumes using the primers ND4F 5'-CGTAGGAGGAGCAGCTATATT-3' and ND4R2 5'-AAGGCTCATGTTGAAGCTCC-3' and conditions in Fonseca et al (2001). Fragments were sent to GenScript (Piscataway, NJ) for cycle-sequencing. ND4 sequences of NF specimens were then compared to known sequences for *Cx. pipiens*, *Cx. quinquefasciatus*, *Cx. pipiens pallens*, *Cx. australicus*, *Cx. restuans*, *Cx. salinarius*, *Cx. torrentium* and *Cx. pervigilans* using Sequencher 4.9 (Gene Codes, Ann Arbor MI).

2.4 RESULTS

Culex specimens were collected from 4 oviposition traps within the city of St. John's, NF between late August and mid-October 2013. From the larvae collected we obtained 18 *Culex* spp. adults and a large number of *Aedes japonicus* adults. *Culex* specimens collected during 2012 (1) and 2013 (18), along with 10 specimens retained from the 2004 (4) and 2005 (6) west coast collections were identified morphologically as *Cx. pipiens*/*Cx. restuans*. Specimens identified by the National Identification Service (B. Sinclair - Agriculture and Agri-Food Canada) were deemed consistent with *Cx. pipiens* morphology but were identified as *Cx. pipiens*/*Cx. restuans* by Dr. Connelly. Thus, relying solely on taxonomic keys was insufficient to allow clear species identification for these specimens. We extracted DNA from 19 of the available 29 specimens and performed the aforementioned three PCR protocols.

After optimization using the positive controls we obtained the expected sized DNA amplification bands with the Crabtree test (Crabtree et al. 1995; *Culex pipiens*/*Cx. quinquefasciatus* – 698 bp, *Cx. restuans* – 506 bp). We found that all specimens from the Island of NF (19 specimens from all four local collections) aligned with the 698 bp band (Fig. 2) indicating they are members of the *Culex pipiens* complex. Likewise, results for the positive controls in the Ace-locus test showed the expected band sizes (*Cx. pipiens* – 610 bp, *Cx. quinquefasciatus* – 274 bp; Smith and Fonseca 2004) and all specimens from NF that successfully amplified using this test (St. John's: 2012 - 1, 2013 - 7; Deer Lake: 2004 - 1, 2005 – 1) aligned with the *Cx. pipiens* band size of approximately 610 bp. Although only two specimens from the west coast (2004-2005) amplified (Fig. 3.) with the Ace-locus assay, examination of ND4 sequences matched sequences of *Cx. pipiens*/*Cx. quinquefasciatus* (Genbank accession nos. KX709951-KX709962). While the mitochondrial ND4 locus is not informative enough to

separate the two species it does rule out the possibility that these specimens were in fact another closely related species or of some other yet unidentified species. Finally, results using the Bahnck and Fonseca (2006) assay showed that most *Cx. pipiens* specimens collected were *Cx. pipiens* f. *pipiens* (St. John's: 2012 - 1, 2013 - 7; Deer Lake: 2004 – 3, 2005 – 6) although one specimen from Deer Lake showed a match for the *Cx. pipiens* f. *molestus* band (lane 6, 284 bp, Fig. 4).

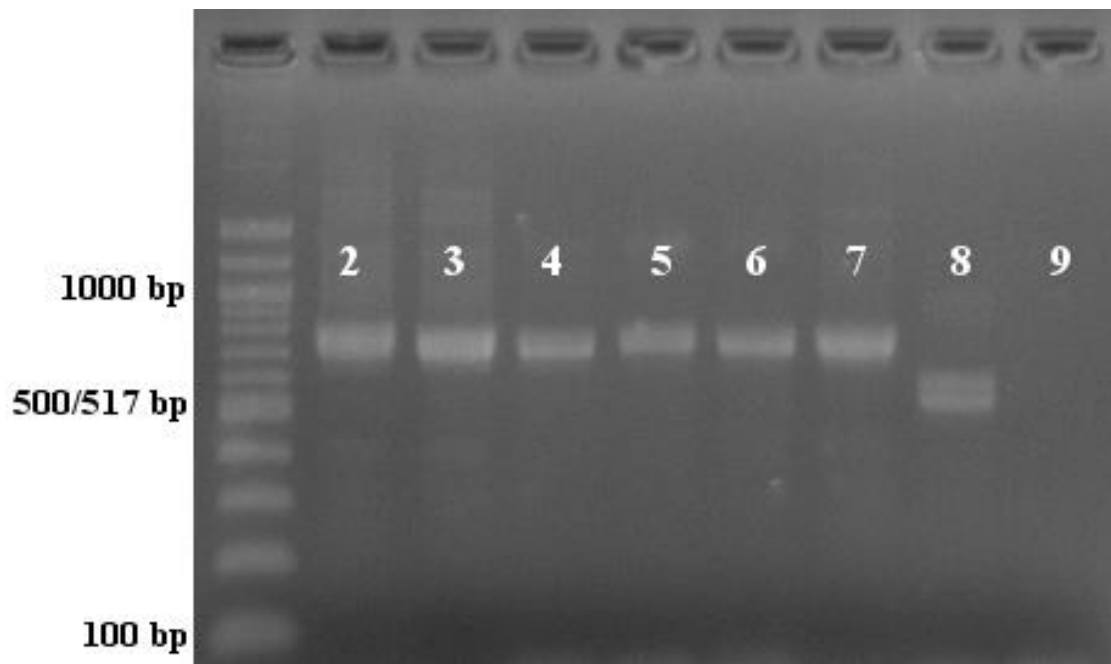


Figure 2-2: Image of 1.5% agarose gel showing variation in size fragments between specimens analyzed with the Crabtree et al. (1995) test. Specimens include three positive controls beside a subset of analyzed local specimens and are ordered as follows: 1 – 100-basepair ladder, 2 – *Culex quinquefasciatus* positive control, 3 – *Culex pipiens* positive control, 4 – *Culex pipiens/restuans* (2004), 5 – *Culex pipiens/restuans* (2005), 6 – *Culex pipiens/restuans* (2012), 7 - *Culex pipiens/restuans* (2013), 8 – *Culex restuans* positive control, 9 – Negative Control.

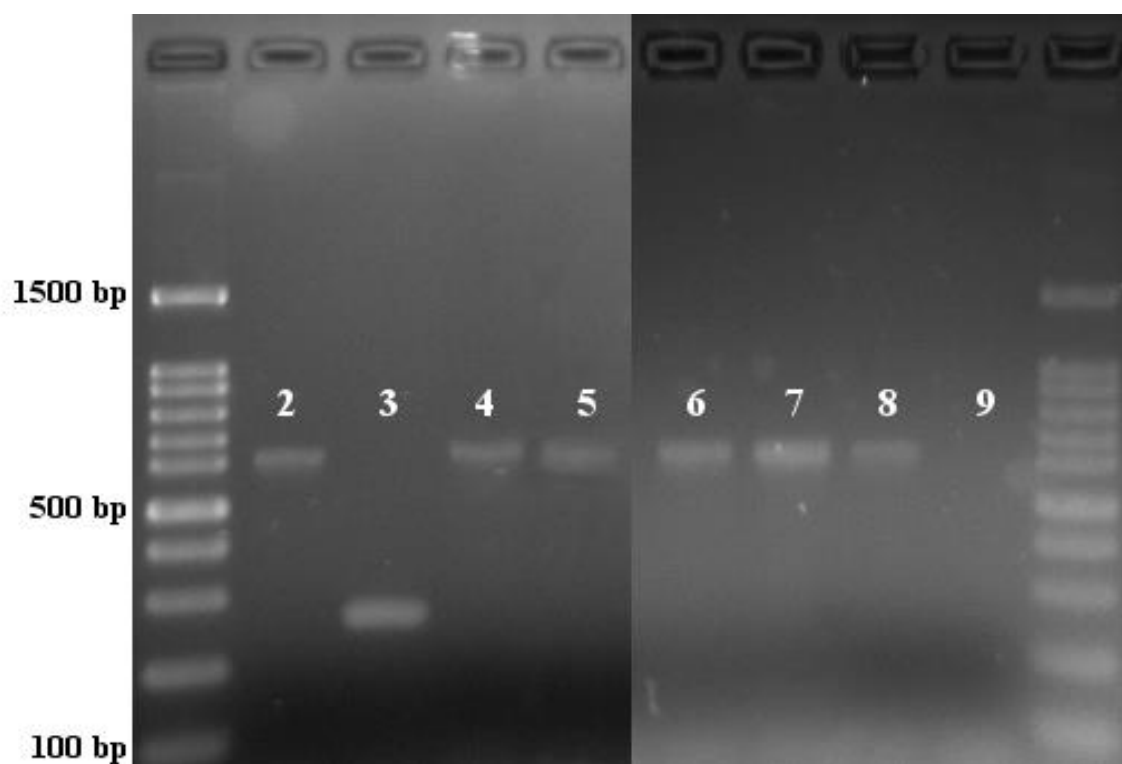


Figure 2-3: Image of 1.5% agarose gel showing variation in size fragments between specimens analyzed with the ACE-locus test (Smith and Fonseca, 2004). Specimens include two positive controls beside a subset of analyzed local specimens and are ordered as follows: 1 – 100 bp Ladder, 2 – *Culex pipiens* positive control, 3 – *Culex quinquefasciatus* positive control, 4 – *Culex pipiens/restuans* (2004), 5 – *Culex pipiens/restuans* (2005), 6 – *Culex pipiens/restuans* (2012), 7 - *Culex pipiens/restuans* (2013), 8 – *Culex pipiens/restuans* (2013), 9 – Negative Control, 10 – 100 bp Ladder. Figure was arranged by means of a double-row electrophoresis gel where only the relevant lanes were retained.

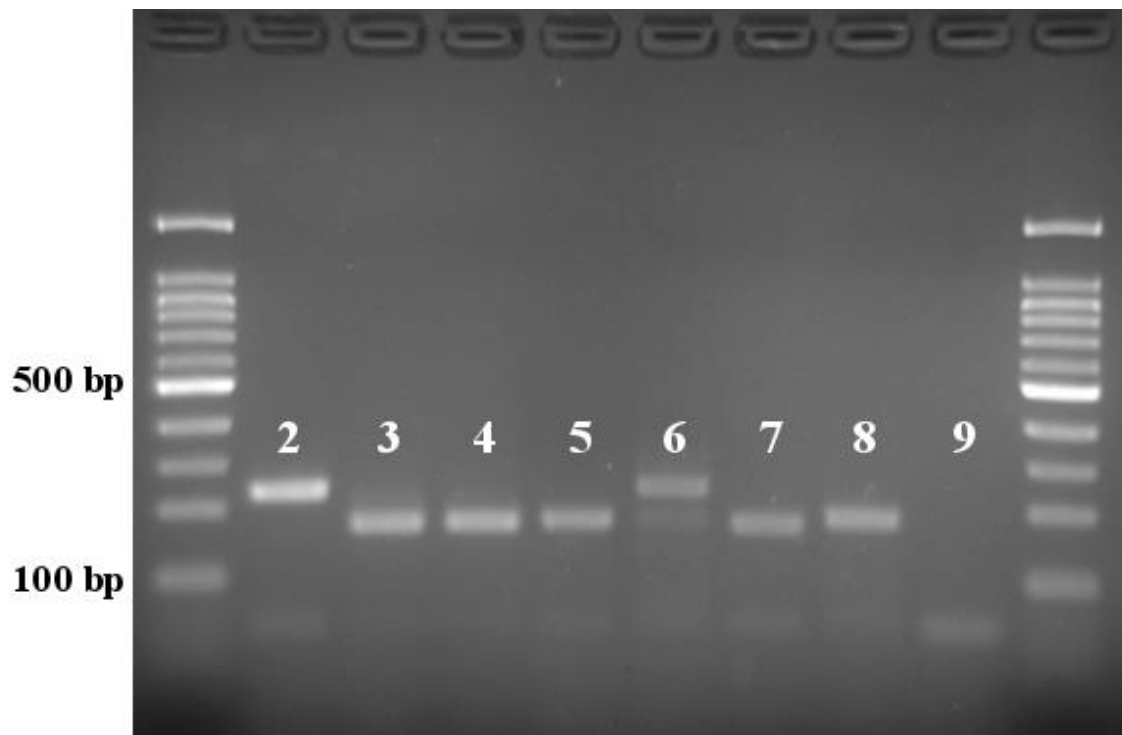


Figure 2-4: Image of 2% agarose gel showing variation in size fragments between specimens analyzed with the *Culex pipiens/molestus* test (Bahnck and Fonseca, 2006). Specimens include positive controls beside a subset of unknown local specimens and are ordered as follows: 1 – 100 bp Ladder, 2 – *Culex pipiens* positive control*, 3 – *Culex pipiens/restuans* (2013), 4 – *Culex pipiens/restuans* (2013), 5 – *Culex pipiens/restuans* (2004), 6 – *Culex pipiens/restuans* (2004), 7 - *Culex pipiens/restuans* (2005), 8 – *Culex pipiens/restuans* (2005), 9 – Negative Control, 10 – 100 bp Ladder.

*** *Culex pipiens* positive control showing a CQ11 band characteristic of form *molestus* (Bahnck and Fonseca, 2006).**

2.5 DISCUSSION

Using both morphological and molecular genetic techniques, collections of *Cx. pipiens* s.s. are now confirmed at two sites on the Island of NF, one on the west coast (specimens collected in 2004 and 2005) and one on the east coast in the St. John's region, collected in 2012-2013. Our examination of approximately two thirds of all available specimens shows clear support for our claim that this species is present on the Island of NF. These analyses indicate that specimens sampled in NF appear to be mixtures of the two forms of *Cx. pipiens* since one specimen showed both diagnostic bands (see Fig. 4, lane 6). This result supports earlier findings that, unlike their northern European counterparts, North American populations of *Cx. pipiens* are a mix of the two forms of *Cx. pipiens* even at much higher latitudes than previously studied (Fonseca et al. 2004). This is interesting since the presence of a signature of a non-diapausing form that is also associated with mammalian feeding may hold important implications for the potential of this mosquito to transmit viruses to humans on the island (Turell 2014).

The low number of adult specimens from our collections in St. John's might lead to the interpretation that this population is a temporary incursion to the city. While the total number of adults reared in our study was not large, they were collected over two years (2012, 2013), primarily from oviposition traps, indicating established breeding populations. Notably, these same oviposition traps produced substantial numbers of *Ae. japonicus* mosquitoes (Fielden et al. 2015). This species is known to out-compete *Cx. pipiens* at breeding sites (Lorenz 2012, Kaufman et al. 2012), so the establishment of a persistent population of *Cx. pipiens* in St. John's warrants further monitoring. That investigation could focus on population size, genetic diversity, and connectivity between populations both locally and abroad to more carefully address the assertion that an established population of *Cx. pipiens* exists in St. John's.

Although *Cx. pipiens* is present on the Island of NF, at least in two general locations, the absence of collection of this species in the region prior to 2004 suggests that it may be a recent arrival to the island. Interestingly, range maps in a number of publications (e.g. Harbach, 2012) indicate the presence of this species in southern Labrador (Fig. 2.1). If the existence of those populations can be confirmed, it could hold important implications concerning population connectivity between the island of NF and mainland Canada. Additionally, the discovery of *Cx. pipiens* in this region and that of a second container-inhabiting mosquito, *Ae. japonicus* (Fielden et al. 2015), a species that has recently been implicated as a vector of La Crosse virus (Harris et al 2015), further demonstrates the importance of consistent monitoring of these medically important insects. Our study shows that *Cx. pipiens* is potentially in contact with a greater proportion of the population of NF than originally thought and warrants a renewed look into potential disease risk on the Island. Such a study should include an examination of population connectivity (between *Cx. pipiens* populations on the island of NF, and between neighbouring regions of mainland Canada), as well as other extrinsic factors known to have a significant effect on the transmission of mosquito-vectored disease (e.g. host density, climate; Semenza and Menne 2009).

Hongoh et al. (2012) modeled the potential range expansion of *Cx. pipiens* in Canada based on projected habitat suitability under climate change. This model predicted our 2012 collection in St. John's, however the climate in more northwestern regions, where collections of this species were made (Hustins 2006, Fig. 2.1), was not predicted to become suitable until much later (2040s). Of note, invasive mosquitoes may be capable of adapting to changing habitat pressures more quickly than is typically assumed in climatic modeling (Egizi et al. 2015). We

assert that further investigations of the NF populations of *Cx. pipiens* have the potential to contribute to an understanding of the forces that may determine vector movements.

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Candidate was the primary author who, along with author 5, contributed to the development of the main questions and research goals addressed in this chapter. The candidate, together with authors 4 and 5 contributed to its formalization and methodologies. Author 2 aided in specimen collection and identification; author 3 provided assistance in obtaining necessary equipment and funding for this project. The candidate, with advice from author 4, was responsible for performing both molecular and data analyses with the exception of a single analysis of ND4 sequence data (author 4). The candidate was solely responsible for the completion of the initial draft of the manuscript, from which all authors were provided the opportunity to assist with refinement and presentation. The author was responsible for preparation of the final draft presented in this thesis.

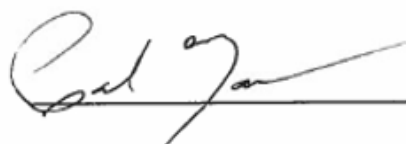
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8-3-17

3. GENETIC CHARACTERIZATION OF *Aedes (Hulecoeteomyia)*

Japonicus (Diptera: Culicidae) in Insular

Newfoundland

3.1 ABSTRACT

Aedes (Finlaya) japonicus (Theobald), the Asian bush mosquito, is a highly invasive mosquito species implicated in the transmission of a number of important human disease-causing pathogens. While innocuous in its native habitat, *Ae. japonicus* is an aggressive container breeding mosquito in its invasive range. While the established range of this invasive species, which expanded in 2001 to include Canada, is now well documented, questions regarding connectivity between established and newly founded populations remain. Here we examine, using population genetic techniques, a newly reported population of *Ae. japonicus* in St. John's, Newfoundland. A panel of 5 microsatellite loci in concert with a mitochondrial marker (ND4) was used to characterize this population alongside populations in other regions of Canada and Europe. Results indicate a level of genetic diversity within the recently discovered Newfoundland population comparable to populations in other regions and a general lack of structure between Canadian populations and those in Europe.

3.2 INTRODUCTION

The movement of human disease vectors – typically small blood-feeding arthropods - directly influences disease risk (Reiter 2010). While proficiency in monitoring established populations of vector species has led to success in tracking the movement of these animals *a posteriori*, the biological characteristics of these animals (e.g. small size, meagre habitat requirements) make their global movements inherently difficult to anticipate or prevent. Furthermore, extrinsic factors such as climate change and more direct anthropogenic influences (e.g. travel, transportation of goods) often complicate and exacerbate the movements of these animals and thus the risk associated with them (Kilpatrick & Randolph 2012). Subsequently, efforts to predict or even detect these initial introduction events remains problematic. Genetic analyses have been used to reveal relationships between populations and help clarify the pathways of movement that these invasive animals use (Egizi et al. 2015; Fonseca et al. 2001, 2010; Zielke et al. 2014, 2015, 2016). Here we focus on a population of the recently discovered invasive mosquito, *Aedes* (Finlaya) *japonicus* (Theobald) (Diptera: Culicidae) on the eastern Canadian Island of Newfoundland (NF).

Ae. japonicus, the Asian bush mosquito, is an invasive mosquito vector species in North America and Europe. This species is one of four within the *Ae. japonicus* species complex whose native range extends along the Ryuku Archipelago and neighbouring regions of eastern Asia (Tanaka et al. 1979). A previous study has found that this species does not travel far within its native range (Fonseca et al. 2001), yet *Ae. japonicus* has spread prolifically since its introduction to North America (1998) and Europe (2002), and has been reported in 33 U.S. states (including the Hawaiian archipelago), 6 Canadian provinces, and 7 European countries to date (Fonseca et al. 2001, Kaufman and Fonseca 2014, Zielke et al. 2015). Due to a high degree of genetic

diversity between the *Ae. japonicus* subspecies and a sister species, *Aedes (Finlaya) koreicus* (Edwards), the taxonomic ranking of individuals within these species has come into question (Cameron et al. 2010). Here I will be following one of the suggestions of Cameron and colleagues (2010) in which the subspecies of *Ae. japonicus s.l.* are raised to the species level; thus the use of *Ae. japonicus* refers to the previous form *Ae. j. japonicus*.

In Europe, *Ae. japonicus* was first detected in a shipment of used tires in northwestern France in 2000 (Schaffner et al. 2003). Though efforts to eradicate this initial introduction were successful, a second European introduction, again associated with used tires, was detected in Belgium in 2002 (Versteirt et al. 2009). This introduction eventually led to the first recognized population of *Ae. japonicus* in Europe. Further investigation revealed very limited spread thought to be linked to low genetic diversity (Versteirt et al. 2009, Zielke et al. 2014). *Ae. japonicus* populations have since been identified in northern Switzerland, southern and northern Germany, Austria, Slovenia, northern Croatia, and, most recently, the Netherlands (Becker et al. 2011, Werner & Kampen 2013, Seidel et al. 2012, Zielke et al. 2015, Ibáñez-Justicia et al. 2014).

Within North America, *Ae. japonicus* individuals were first collected in New York, New Jersey, and Connecticut in 1998 (Peyton et al. 1999, Munstermann & Andreadis 1999). This subspecies spread rapidly in North America, and by 2000 adult *Ae. japonicus* were collected along the Quebec-Vermont border (Franklin, QC.; Savignac et al. 2002). Collections of adult *Ae. japonicus* were made the following year (2001) in the Niagara region of southern Ontario (Thielman & Hunter 2006). Though its expansion in Canada has proceeded slowly, compared to its initial spread in North America, *Ae. japonicus* is now present in all eastern and Atlantic Canadian provinces with the exception of Prince Edward Island (potentially due to a lack of surveillance; Carolyn Sanford pers. comm). This species was collected in St. John's and the

surrounding region for the first time in the fall of 2013 (Fielden et al. 2015). Notably, Jackson and colleagues (2015) also reported the first collection of *Ae. japonicus* in western Canada (Vancouver, British Columbia). While a connection between eastern populations and the BC collection has not been formally evaluated, the BC collection likely represents a separate introduction into Canada with possible connections from northwestern US populations, Asia, or elsewhere (Jackson et al. 2015).

The medical significance of *Ae. japonicus* makes acute an understanding of how this species moves throughout its invasive range. While not considered medically important or a nuisance biter in its native range, *Ae. japonicus* is an aggressive biter that readily feeds on humans in Europe and North America (Tanaka et al. 1979, Kampen and Werner 2014, Molaei et al. 2009). Invasive populations of this mosquito have the potential to transmit important zoonotic pathogens in the New World. Field-collected *Ae. japonicus* specimens, for example, have repeatedly tested positive for West Nile virus (WNV) implying natural feeding on bird species (Kaufman and Fonseca, 2014). Blood meal analyses and reports of nuisance biting demonstrate a proclivity to feed on a wide variety of human hosts (Kaufman and Fonseca, 2014). This suggests a potentially significant role as a potential bridge vector; that is, this mosquito shows a proclivity to feed on a variety of hosts including both birds and mammals including humans. In addition to WNV, laboratory analyses have confirmed that *Ae. japonicus* is a potential vector of several human pathogens (Sardelis et al. 2002, Turell et al. 2013, Sardelis et al. 2003, Takashima & Rosen 1989, Schaffner et al. 2011). Notably, Harris and colleagues (2015) identified *Ae. japonicus* as a significant vector of La Crosse virus (LACV) in the American Appalachian region. LACV is an arbovirus typically spread in North America by the eastern tree hole mosquito, *Aedes*

triseriatus (Harris et al. 2015). This arbovirus can cause arboviral encephalitis that is particularly dangerous to children (McJunkin et al. 2001).

A number of studies have used population genetic techniques in order to attempt to identify and understand patterns of migration and range movements of populations of *Ae. japonicus*. Fonseca and colleagues (2001) used random polymorphic amplified DNAs (RAPDs) and mitochondrial loci (ND4) to investigate populations of this species in the United States. Though this study found evidence of multiple introductions to North America, in addition to genetic similarity between some American and Japanese samples, no conclusive statement could be made regarding source populations. Fonseca and colleagues (2010) extended on this initial analysis and identified patterns of genetic diversity that demonstrate at least two separate introductions of *Ae. japonicus* into the eastern United States. A repeated analysis in the following years revealed that this distinction was being lost as these independent populations mixed and spread further throughout North America (Fonseca et al. 2010).

Similar to the North American studies, Zielke and colleagues (2014) used mitochondrial (ND4) and microsatellite loci and found evidence of at least two introductions into Germany. Unlike the North American introductions that were separated geographically, two temporally separate introductions of genetically distinct *Ae. japonicus* took place within the same region of western Germany (Bonn). Zielke and colleagues (2014) also found that populations in Belgium, Switzerland, and Austria/Slovenia were closely related and thus likely originated from the same source population; either as a single event that seeded subsequent populations, or, less likely, as up to three separate introductions from Asia, or elsewhere. Zielke and colleagues (2015) analyzed north German and Dutch populations in relation to previously analyzed European populations. This study found that while north German populations likely originated from a

persistent northward spread of populations from west Germany, Dutch populations were notably diverse relative to other European populations. This finding supported the possibility that populations in the Netherlands originated from a separate introduction of a large number of genetically diverse individuals from overseas (Zielke et al. 2015). Importantly, studies of both the North American and European *Ae. japonicus* invasions have suggested that multiple introductions, resulting in genetically diverse admixed populations, have enabled this species to become highly invasive in its exotic range (Fonseca et al. 2010, Zielke et al., 2014).

While the expansion of *Ae. japonicus* in eastern Canada was predicted, the introduction to the Island of NF does represent an expansion across significant boundaries to direct migration and introduction points to major shipping ports and international airports. We use population genetic techniques to characterize a recently described population of *Ae. japonicus* in St. John's (NF), and investigate potential connectivity between this population and those outside our province. In order to provide insight into possible pathways of introduction for this species, we compared members of a population from St. John's with those of two other Canadian populations in Nova Scotia and Ontario. In addition, we compare Canadian populations to those in Europe in order to assess the possibility of a novel introduction to our region.

3.3 MATERIALS AND METHODS

3.3.1 Sources of *Aedes japonicus* samples

Aedes japonicus specimens were obtained from 3 primary sources. Firstly, 47 specimens were collected in and around east St. John's, NL, Canada in the early spring of 2013. These specimens were caught using 27 artificial oviposition traps covering an urban area of approximately 4 km² (see Fielden et al. 2015; Appendix III). Mosquito larvae from these traps were then reared to adulthood using commercial mosquito breeders (BioQuip, Rancho Dominguez, CA, Item #1425). Adults were freeze killed (at -20°C for 24 hours) and pinned prior to identification. Secondly, specimens collected throughout this species' range in Ontario were obtained through a partnership with Brock University (St. Catharines, Ontario). These samples were collected throughout 20 of the 36 health units (covering a range of latitudes between 42.101130 and 56.848899) that divide the province of Ontario for the purpose of organizing health care in the province. Collection efforts in Ontario were undertaken either as part of Public Health Ontario's WNV program or as part of a similar program run by Health Canada's First Nations and Inuit Health Branch. As a result, samples provided were either whole frozen adults, or slurries of one or more individuals (see below). A subset of 75 samples was selected using both whole adults and slurries of single specimens stored frozen in BA-1 diluent (1× Medium 199 with Hanks' balanced salt solution, 0.05 M Tris buffer [pH 7.6], 1% bovine serum albumin, 4.2 mM sodium bicarbonate, 100 µg of streptomycin per liter, 1 mg of amphotericin B per liter). Specimens from these collections were selected such that all 20 health units were represented. Whole specimens were used whenever possible; however, in regions where none were available, slurries of single mosquitoes were selected. Thirdly, 12 samples from two locations in Nova

Scotia were provided by the Nova Scotia Department of Natural Resources; five specimens collected in Coldbrook County (45.061128, -64.598240) and 7 specimens 120 km northeast in Colchester County (45.448954, 63.168695). These shared samples were collected by Dr. Murray Colbo and Natural Resources personnel from two small pools of water in residential areas (J. Ogden, pers. comm). Finally, in order to calibrate microsatellite data from Zielke et al. (2015), DNA from 10 European *Ae. japonicus* samples were shipped to Memorial University, amplified, and processed alongside Canadian samples. This allowed the inclusion of microsatellite data from a total of 406 European samples (collected from 11 sampling regions in the Netherlands, Germany, and Slovenia).

3.3.2 Specimen Identification

Specimens from Newfoundland and Nova Scotia were identified using standard taxonomic keys (Darsie and Ward 2005, Thielman & Hunter 2007) and to species as per Cameron and colleagues (2010). Specimen identifications were corroborated by both the National Identification Service program (Det. B. Sinclair) offered by the department of Agriculture and Agri-Food Canada (one specimen was retained for the national collection) and by Dr. Roxanne Connelly at the Florida Medical Entomology Laboratory (Vero Beach, Florida). Specimens collected in Ontario were identified as part of established mosquito monitoring programs and, therefore, identifications were not repeated by the authors here. Vouchers of the *Ae. japonicus* St. John's collection are retained at Memorial University, Science Building room 4113.

3.3.3 DNA Extraction

We extracted DNA from a total of 108 whole adult specimens and from 36 mosquito slurries. Each intact specimen was photographed dorsally and laterally using a Leica DFC420 digital microscope camera mounted on a Leica MZ95 compound microscope. Photographs of individual specimens allow for checks of identification if aberrant or absent genotyping results are obtained. Whole mosquitoes were macerated for DNA extraction using 100 μ L pipette tips that were formed into a pestle by briefly melting the ends of the pipette tips in a gas flame. Extractions were performed using the Qiagen DNeasy Blood and Tissue kit and provided protocol (DNeasy Tissue Kit, Qiagen, Valencia, CA). DNA extractions from single specimen slurries were performed using a modified version of this protocol in which a 200 μ L aliquot of vortexed mosquito slurry replaced the initial addition of buffer ATL and proteinase K. DNA concentration was quantified and overall quality of the extracts was evaluated using a Nanodrop 1000 spectrophotometer and extracted DNA was stored at -20 °C until needed for the amplification step (see below) and for long-term storage.

3.3.4 Microsatellite Amplification

Two multiplex PCR setups (A & B; modified from Widdel et al. 2005) were used to amplify a panel of 5 microsatellite loci for 134 Canadian *Ae. japonicus* from Ontario (75), Nova Scotia (12), and Newfoundland (47). 10 European DNA samples were analyzed alongside Canadian samples in order to calibrate data from a recent analysis of European *Ae. japonicus* (406 individuals from 11 collection locations; Zielke et al. 2015). Multiplex A reaction mixtures contained 10 μ L GoTaq Colourless Mastermix, 6.9 μ L nuclease free (nf) H₂O, forward and reverse primers for 2 loci (OJ5 and OJ10 at 0.2 μ M per primer) and 1.5 μ L template DNA.

Multiplex B reaction mixtures comprised of 10 μ L GoTaq Colourless Mastermix, 6.1 μ L of H_2O , forward and reverse primers for 3 loci (OJ70, OJ187, and OJ338 at 0.2 μ M per primer) and 1.5 μ L template DNA. Samples that did not amplify at all loci were identified and these failed reactions were repeated separately from the multiplex. PCR amplifications were carried out on a Mastercycler ep gradient S (Eppendorf, Mississauga, Ontario) using conditions reported by Widdel and colleagues (2005). All completed PCRs were sent to The Centre for Applied Genomics (TCAG) at the Hospital for Sick Children (Toronto, Ontario) where they were analyzed on an ABI 3730 DNA Analyzer (Applied Biosystems, Burlington, Ontario). Size calling of raw data was performed at Memorial University using GeneMapper® Software.

3.3.5 ND4 Sequencing

Following Egizi and colleagues (2015), we amplified a 424 bp section of the mitochondrial NADH dehydrogenase subunit 4 (ND4) gene. This protocol uses a reverse primer modified from the original protocol reported by Fonseca et al. (2001) in order to better fit this region in *Ae. japonicus* (ND4R1X - 5'-TGATTGCCTAAGGCTCATGT-3'; Egizi et al., 2015). PCR conditions followed Fonseca and colleagues (2001), with a 10 minute denaturation step at 96°C preceding 35 cycles of 40 s at 94°C , 40 s at 56°C , and 60 s at 72°C , and a 7 min final extension at 72°C. PCR products were visualized by gel electrophoresis using RedSafe nucleic acid staining solution (iNtRON Biotechnology Inc., Sungnam-Si, Gyunggi-Do). Samples that amplified were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA) with a modified protocol that substitutes DNase free H_2O for the provided elution buffer (as required by the DNA sequencing facility). Amplicons were sequenced bi-directionally at TCAG. Forward and reverse sequences were combined using BioEdit v. 7.2.5 to produce FASTA files for each

specimen (Hall 1999). These files were aligned using MEGA v. 6.0 to identify nucleotide polymorphisms (Tamura et al. 2013).

3.3.6 Statistical Analysis

Nei's genetic distance ($G'st$; Hedrick 2005) and pairwise population F_{ST} values were calculated and a principal coordinate analysis (PCoA) was performed ($G'st$) using GenAlEx v. 6.501. a (Peakall & Smouse 2012). This distance measure was chosen since it is a standardized measure of Nei's Gst (Nei 1973) that describes the average differentiation observed over multiple loci after correction for limitations observed with the original measure. Potential departures from Hardy-Weinberg equilibrium were also investigated using GenAlEx v.6.501 and Micro-Checker v. 2.2.3 was used to investigate potential null alleles, miscalls due to stuttering, and large allele dropout (Dieringer & Schlötterer 2003, Oosterhout et al. 2004). Additionally, measures of genetic diversity were calculated using HP-Rare v. 1.1 (rarefied allelic richness - A_r , Kalinowski 2004) and GenAlEx v. 6.501 (remaining diversity measures – see Table 3-1; Peakall and Smouse 2006, 2012).

Bayesian cluster analyses were performed using the program STRUCTURE 2.3.4 (Pritchard et al. 2000). This analysis was performed once using microsatellite signatures from all samples, then again using only Canadian samples. The first analysis using all individuals was performed using $K = 1$ to $K = 10$, with 10 independent runs at each value of K . The second analysis (Canadian samples only) was performed using $K = 1$ to $K = 5$ with 15 independent runs at each value of K . All STRUCTURE analyses were run with 10,000 burn-in steps and 50,000 runs using a model of independent allele frequencies, allowing for admixture, and using the LOCPRIOR parameter. Results from these analyses were subsequently evaluated using the web-

based program STRUCTURE HARVESTER that determines the optimal number of clusters (K) using the method of Evanno and colleagues (2005; Earl & vonHoldt 2012). Results from STRUCTURE runs using this ideal K were then extracted and processed using two programs (CLUMPP and DISTRUCT; Jakobsson 2007, Rosenberg 2004) to produce bar plots from STRUCTURE's output. Individual Q scores provided by STRUCTURE have been used recently to estimate nuclear ancestry associated with mitochondrial haplotype (Egizi et al. 2016). These scores are numerical estimations of the proportion of each individual's genetic ancestry linked to each cluster. In order to investigate a potential trend in this association within each Canadian population, \bar{Q} scores from 10 runs were averaged across individuals and within populations.

Additional investigations of genetic structure using non-Bayesian methods were conducted using the R package *Poppr* (version 2.2.1; Kamvar et al. 2014). This package allows the user to designate the level (or strata) considered for each analysis (e.g. continent, country, collection site). Using this package, an analysis of molecular variance (AMOVA) and an assessment of potential linkage-disequilibrium between countries (Europe) or provinces (Canada) was performed. Additionally, a minimum spanning network using Bruvo's genetic distance was produced. Bruvo's genetic distance utilizes a stepwise mutation model appropriate for microsatellite markers (Bruvo et al. 2004).

3.4 RESULTS

A check of Hardy-Weinberg equilibrium (HWE) showed significant deviation at two loci from both the Ontario (OJ5 & OJ70) and Newfoundland (OJ5 & OJ338) populations. As with similar deviations in European populations, there was no evidence of null alleles or miscalls due to stuttering or large allele dropout when evaluated with Micro-checker. However, given the deviation from HWE observed at the OJ5 locus in both Ontario and Newfoundland, this locus was removed and the analyses performed a second time to test whether it had any effect on the observed outcomes. Its removal from the analysis did not have any observable effect on the observed outcomes so the locus was retained. Shannon Diversity (H), rarefied allelic richness (A_r), and other diversity measures (as seen in Table 3-1) show an unexpected level of genetic diversity within the Newfoundland *Ae. japonicus* population.

As expected, using the determined number of genetic clusters (most likely $K = 2$; Evanno's method is unable to assess $K=1$ therefore the K values with the highest $\ln P(K)$ was chosen), results from analyzing European samples alone were identical to those of Zielke and colleagues (2015; Fig. 3-1B). When Canadian samples are analyzed alongside European samples, K remains at two, Canadian samples are clustered along with European samples, and no patterns indicative of population structure are detected within Canadian populations, or between those from Canada and Europe (Table 3-2, Fig. 3-1A). To further investigate this outcome, the structure analysis was performed using only Canadian samples and, again, samples from all three provinces are approximately uniformly mixed between each cluster and display no evidence of population structure (Fig. 3-1). Additionally, probability (\bar{Q}) scores were calculated for each province by calculating the mean \bar{Q} score of each genotype for each individual across 10 runs,

then calculating the mean across all individuals with each province. Both Newfoundland (n=47) and Ontario (n=66) populations exhibit an even split between $\overline{Q}_{\text{genotype1}}/\overline{Q}_{\text{genotype2}}$ (NF: 0.497/0.502, ONT: 0.504/0.496), while Nova Scotia (n = 12) showed a slight deviation in this ratio (0.459/0.541). Interestingly, a deviation is also present for Nova Scotia samples within the principal components plot of G'st values from Canadian and European samples (Fig. 3-2).

Further investigation of population structure using a non-Bayesian approach indicated no structure within or between Canadian populations. An AMOVA showed that most variation between Canadian populations is held within individuals with only 2.16% existing between provinces (Table 3-3). Additionally, a linkage disequilibrium analysis of Canadian populations showed no significant deviation from a randomly generated dataset (Fig. 3-3). Finally, a minimum spanning network using Bruvo's genetic distance shows the same pattern of admixture demonstrated by the STRUCTURE plots in Canadian populations (Fig. 3-4).

Examination of ND4 sequences from 59 Canadian individuals (Ontario - 23, Nova Scotia - 12, and Newfoundland – 24) yielded a total of 5 haplotypes (Table 3-4, Fig. 3-5). Within Ontario populations, haplotypes H1 (35%), H9 (39%), and H12 (26%) were detected. Newfoundland was the most diverse, with haplotypes H1 (63%), H6 (8%), H9 (17%), and H12 (8%) identified. Additionally, one rare allele (H10, <5%) was identified in a single Newfoundland individual. Unexpectedly, all specimens from Nova Scotia were assigned the H1 haplotype. This is surprising given *Ae. japonicus*' known mitochondrial heteroplasmy (multiple mitochondrial haplotypes in a single organism; Zielke et al. 2015). Interestingly, when ND4 haplotypes present in Canadian populations are compared with a recent analysis of nuclear ancestry of this species in the United States, some similarity is present between Newfoundland

and the “New York (NY) nuclear ancestry”, and between Ontario and the “Pennsylvania (PA) nuclear ancestry” (Egizi *et al.* 2016). In their analysis, Egizi and colleagues (2016) identified links between ND4 haplotype and STRUCTURE cluster results based on seven microsatellite loci (five of which were used in this study), indicating that an association between mitochondrial and nuclear (microsatellite) loci is still detectable between the two introduction events first described by Fonseca *et al.* (2010). While this association is not observed in Canadian populations, at least when five of the seven microsatellite markers used in related analyses, it is noteworthy to highlight that the patterns of ND4 haplotypes are similar to those reported by Fonseca and colleagues (2010) and Egizi and colleagues (2016). When compared with populations abroad, four of the five ND4 haplotypes detected in Canadian samples were shared with populations in Europe and all 5 were previously detected in American *Ae. japonicus* populations. While haplotypes H1, H9, and H12 are ubiquitous throughout all invasive *Ae. japonicus* populations, H6 and H10 were detected previously in low numbers in Connecticut, New Jersey and New York populations. Within Europe, H6 has only been detected within a single population in west Germany (Zielke *et al.* 2015).

Table 3-1: Location, sample size, and measures of genetic diversity in Canadian populations.

Pop	N	MLG	eMLG	SE	H	G	lambda	E.5	H _{exp}	Ia	$\bar{r}D$	A _r *
Ontario	75	75	11	0.00E+00	4.32	75	0.987	1	0.724	0.185	0.0466	4.00
Newfoundland	47	47	11	1.18E-06	3.85	47	0.979	1	0.708	0.0744	0.0194	4.01
Nova Scotia	11	10	10	0.00E+00	2.27	9.31	0.893	0.96	0.55	0.6794	0.1766	3.29

Pop = Population name, N = Number of individuals observed, MLG = Number of multilocus genotypes (MLG) observed, eMLG = The number of expected MLG at the smallest sample size ≥ 10 based on rarefaction, SE = Standard error based on eMLG, H = Shannon-Wiener Index of MLG diversity (Shannon, 2001), G = Stoddart and Taylor's Index of MLG diversity (Stoddart & Taylor, 1988), lambda = Simpson's Index (Simpson, 1949), E.5 = Evenness (Pielou, 1975; Ludwig & Reynolds, 1988; Grünwald et al., 2003), H_{exp} = Nei's unbiased gene diversity (Nei, 1978), Ia = The index of association (Brown, Feldman & Nevo, 1980; Smith et al., 1993), $\bar{r}D$ = The standardized index of association (Agapow & Burt, 2001).

* Rarefied allelic richness calculated using the computer program HP-rare (Kalinowski, 2005)

Table 3-2: Results from Structure Harvester (Evanno et al. 2005) showing mean $\text{LnP}(K)$, standard deviation in $\text{LnP}(K)$, and ΔK observed in STRUCTURE analyses for K values of 1-10 in Canadian and European populations (A) and values of 1-5 in Canadian populations only (B). Using this method, K is selected based on the modal value of ΔK over all runs. Bold values signify the optimal level of K in each run of Structure Harvester.

Group	K	Reps	Mean $\text{LnP}(K)$	Stdev $\text{LnP}(K)$	ΔK
A	1	17	-7877.141176	44.928945	—
	2	20	-7440.975000	5.078217	56.814462
	3	20	-7293.325000	17.637098	3.885560
	4	20	-7214.205000	30.756625	3.895583
	5	20	-7254.900000	56.181211	0.120948
	6	20	-7302.390000	214.321969	0.564734
	7	20	-7228.845000	93.598923	0.895897
	8	20	-7239.155000	95.123972	0.099712
	9	20	-7258.950000	82.130778	0.563674
	10	20	-7232.450000	37.680785	—
B	1	15	-1738.600000	0.053452	—
	2	15	-1777.146667	41.684082	5.452761
	3	15	-2042.986667	163.704729	1.628583
	4	15	-2042.220000	166.779483	0.339570
	5	15	-2098.086667	132.025170	—

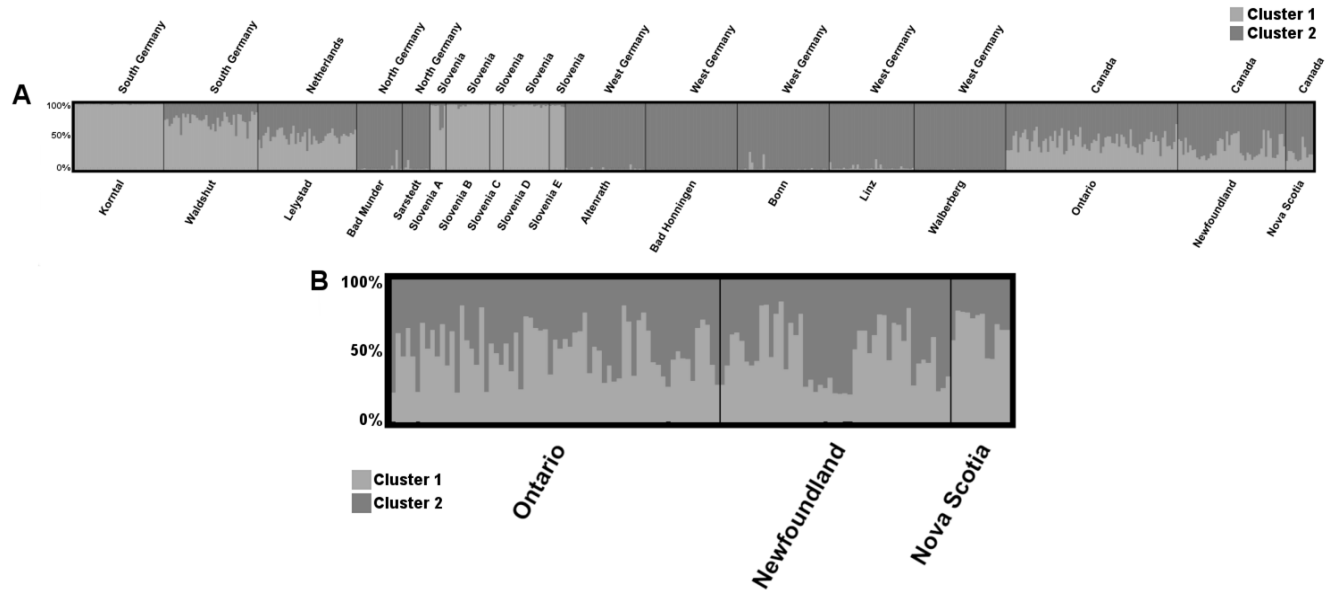


Figure 3-1: Results of two Bayesian cluster analyses using the program STRUCTURE. In this analysis each individual is represented by a set of \bar{Q} -values, probability scores that describe the proportion of a given individual's nuclear ancestry to any particular cluster(s) (Y-axis). The number of clusters ($K = 2$) was determined using Evanno's method for both analyses (Table 3-2; Evanno et al. 2005). Analyses included samples from both European and Canadian populations (A) or from Canadian populations only (B).

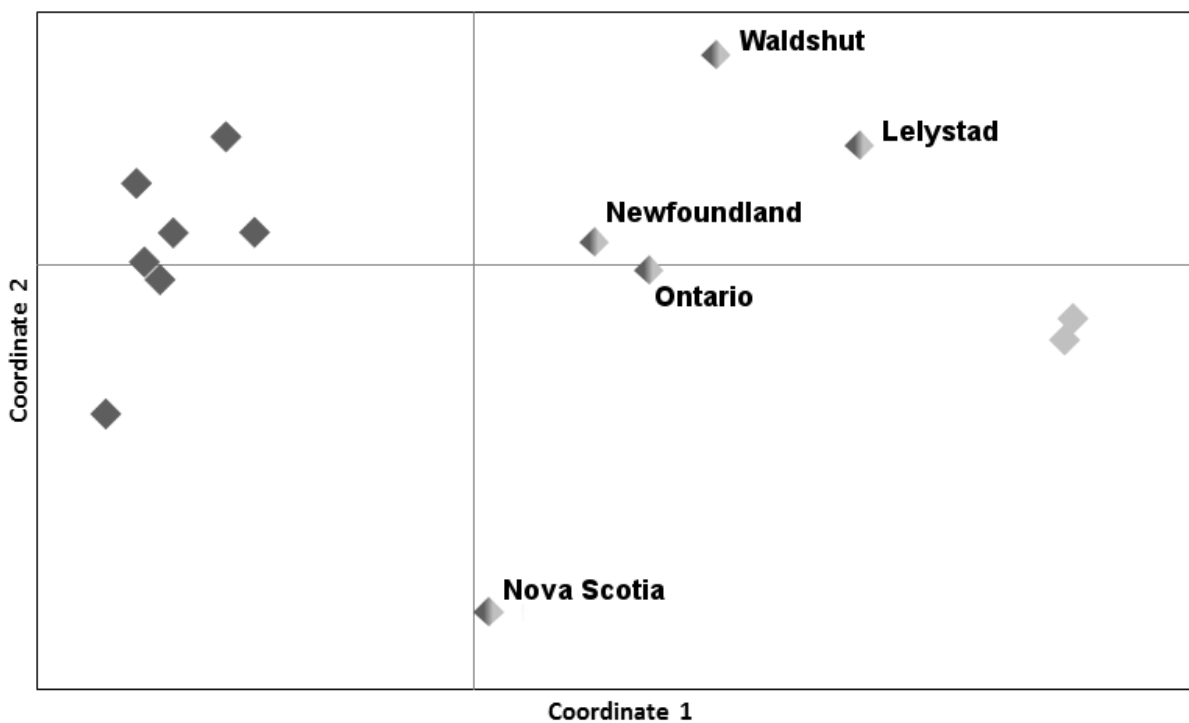


Figure 3-2: Principal coordinate plot of $G'st$ values showing clustering of multi-locus genotypes in north/west Germany (dark grey points), and south Germany/Slovenia (light grey points). Admixed populations (bicoloured points) are labelled independently. Points are coloured based on their assigned cluster in Fig. 3-1(A).

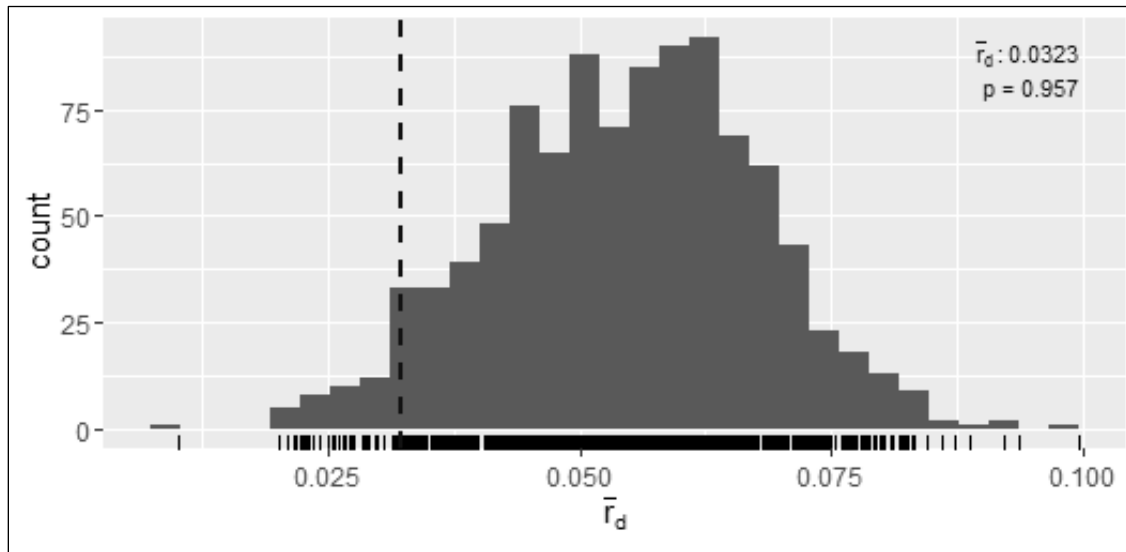


Figure 3-3: Results of a linkage disequilibrium analysis in *Poppr*. The vertical dashed line represents observed values in Canadian populations only and is displayed over histograms showing results of 999 permutations of the same data in which alleles are shuffled at each locus, eliminating any potential linkage that may exist (Kamvar et al. 2014). The index \bar{r}_d describes linkage within a sample while accounting for the number of loci sampled (Agapow & Burt, 2001).

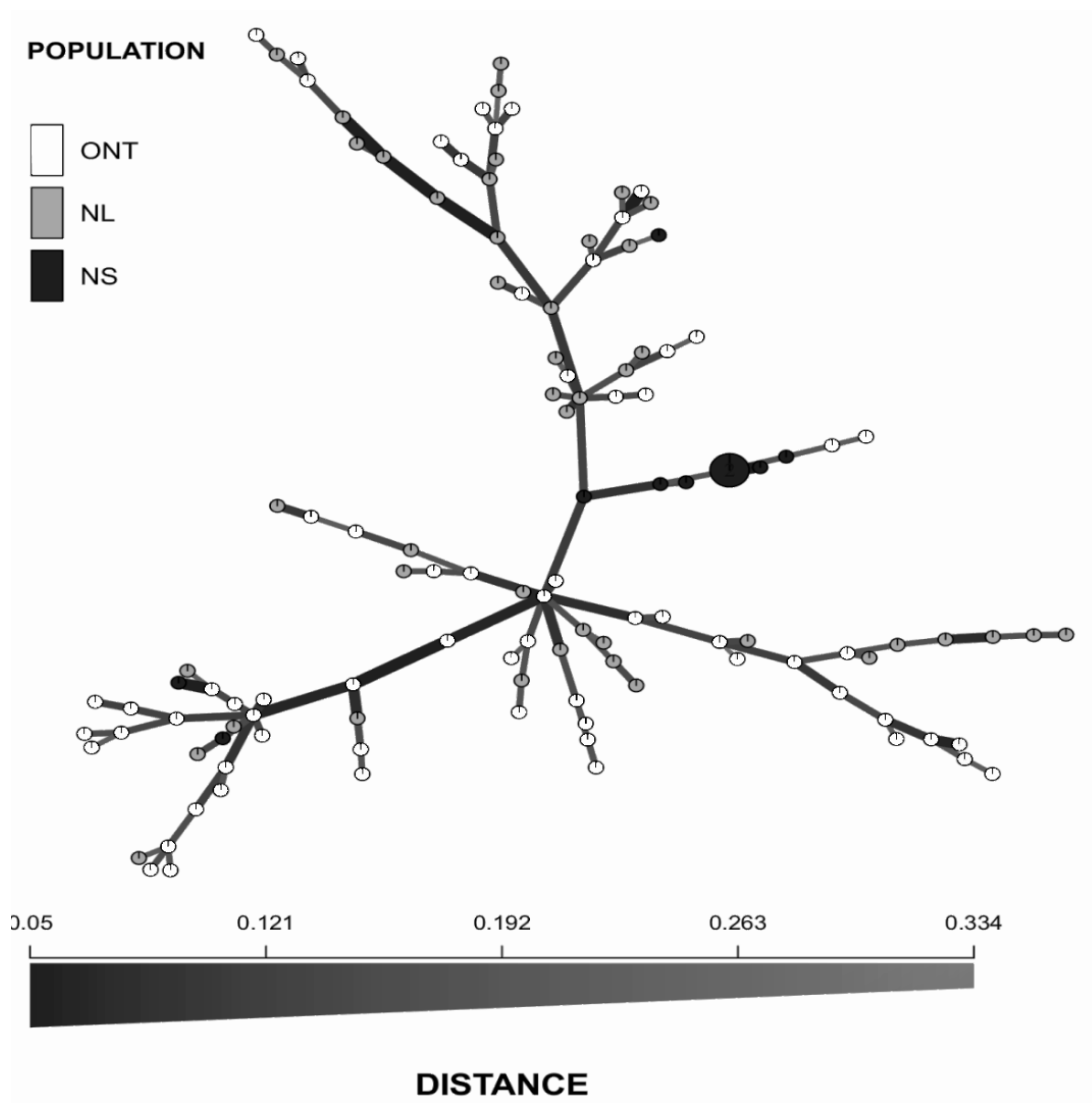


Figure 3-4: Minimum spanning network containing all Canadian samples. Shading of branches indicates Bruvo's genetic distance between samples (Bruvo et al. 2004).

Table 3-3: Results of a hierarchical analysis of molecular variance for populations of *Ae. japonicus* in 3 Canadian provinces (Ontario, Nova Scotia, and Newfoundland).

Source of Variation	Df	Sum Sq	Mean Sq	Percent Variation (%)
Among Provinces	2	6.72	3.36	2.16
Within Provinces	117	149.60	1.28	-9.26
Within Individuals	120	185.52	1.55	107.10

Table 3-4: Collection and ND4 haplotype information for *Ae. japonicus* specimens collected in Canada.

Location	Health Unit*/ Collection Location	n	H1	H6	H9	H10	H12
Ontario	CHK	5	1		1		3
	HAL	4	2		1		1
	HKP	4	2		2		
	KFL	2	1				1
	NPS	2	1		1		
	PDH	1					1
	SMD	1			1		
	SR	1			1		
	SUD	1			1		
	TY	1	1				
	WY	1			1		
Subtotal			8		9		6
Nova Scotia	Coldbrook Co.	7	7				
	Colchester Co.	5	5				
Subtotal			12				
Newfoundland	Downtown St. John's	8	4	2	1	1	
	Center City	16	11		3		2
Subtotal			15	2	4	1	2

*The province of Ontario is divided into health units wherein mosquito collection programs take place. While the provinces of Nova Scotia and Newfoundland and Labrador are divided into health regions, no formal mosquito monitoring programs are carried out.

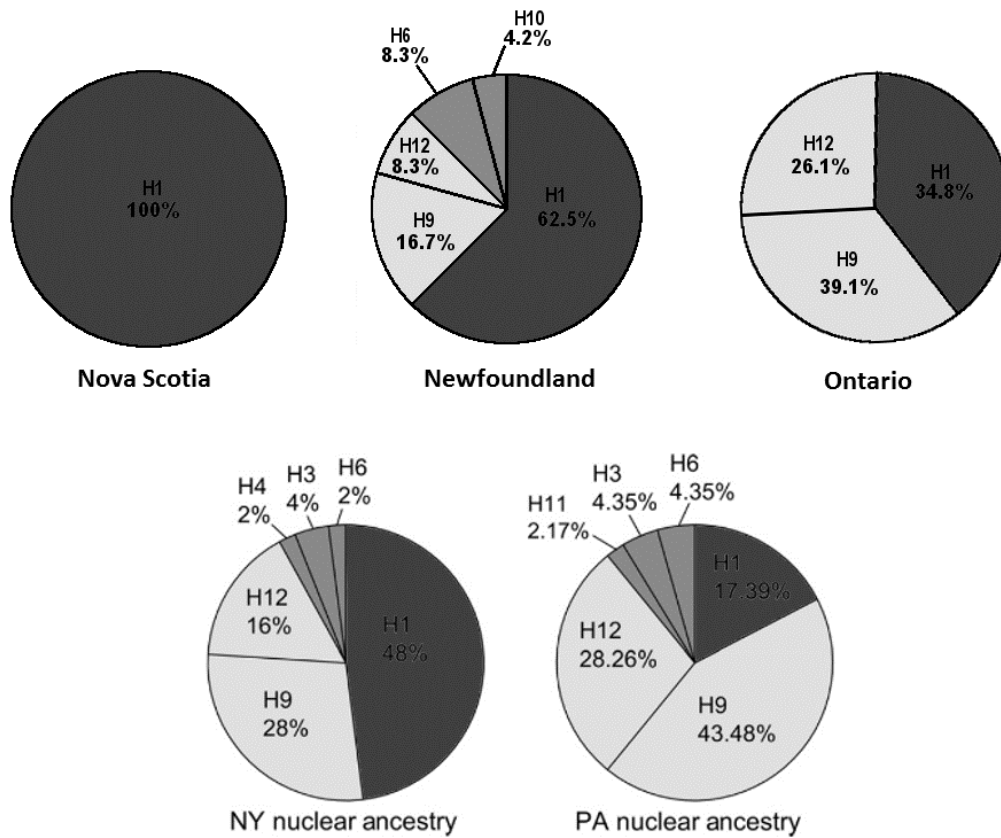


Figure 3-5: Relative frequencies of ND4 haplotypes within *Ae. japonicus* samples analyzed from Ontario (n = 23), Nova Scotia (n = 12), and Newfoundland (n = 24). Populations in each Canadian province are compared to Egizi and colleagues' (2016) analysis of Pennsylvanian (PA) populations in which populations comprised of individuals with mainly haplotype H1 are designated as having New York (NY) nuclear ancestry, whereas of haplotypes H9 and H12 are associated with Pennsylvanian nuclear ancestry. Modified from Egizi et al. (2016).

3.5 DISCUSSION

Aedes japonicus is a highly invasive and medically important mosquito species that has recently been reported in St. John's, the island capital and largest city of the Atlantic Canadian province of Newfoundland and Labrador. While collection records in this province are limited, this species was not detected during survey efforts in 2004-2005, or during 2011-2012. The potential medical impact of this species has emphasized the need to understand how these animals move throughout large-scale geographic space. While long-distance spread of this species is thought to be linked to the transportation of material goods, local spread has been linked to human travel and transportation, especially along motorways (Kaufman et al. 2012, Shaffer et al. 2009, Egizi et al. 2016). Connections between St. John's and Europe are sparse; limited shipping and a small number of international flights travel between St. John's and destinations in Europe (St. John's Airport Authority, 2016). However the movement of European disease-causing organisms across the Atlantic to Newfoundland is not unheard of; two well-known examples include the arrival of caribou brain worm (*Elaphostrongylus rangiferi*) from Scandinavia (associated with imported reindeer in the early 1900s) and French heartworm (*Angiostrongylus vasorum*) first detected in Aquaforte in 1973 (Lankester & Fong 1989; Smith & Threlfall 1973). Thus, while connectivity to Europe cannot be ruled out without testing, Newfoundland's close proximity and high connectivity to mainland Canada suggests the likelihood that this introduction event resulted from a movement from continental North America rather than long distance dispersal event from Europe. Thus, potential links between *Aedes japonicus* in St. John's and populations in mainland Canada and Europe were investigated.

Using Bayesian and traditional population genetic techniques, an analysis using a panel of 5 microsatellite loci has detected no evidence of population structure between populations of

Ae. japonicus on the Island of Newfoundland and those that exist throughout Europe. However, a lack of detectable structure between Newfoundland and other Canadian populations and an absence of unique European ND4 haplotypes (H33, H44, H45) present in Newfoundland's samples (Zielke et al. 2014), seem to suggest that populations of *Ae. japonicus* in Newfoundland have not been influenced by European migrants. Results from non-Bayesian analyses also mirror these findings; no detectable structure was identified in either the linkage disequilibrium analysis or between multi-locus genotypes in Newfoundland and those in other Canadian populations (Fig. 3-3, 3-4). Additionally, AMOVA results indicate that the majority of variability within all three populations exists within individuals, not between provinces (Table 3-3). However samples from other regions in Canada and the United States would be required in order to more directly assess potential connectivity between the remainder of North America and more soundly eliminate the potential for connectivity between Newfoundland and Europe.

The lack of detectable structure between Canadian populations suggests that Newfoundland's population of *Ae. japonicus* likely originated from a continual spread from neighbouring regions in Canada. Additionally, given the level of diversity and admixture observed in Newfoundland individuals, the introduction of this species to the Island was likely quite large in number, or occurred as multiple smaller introductions. Such a scenario would be necessary in order for the diversity of source populations to be represented as comprehensively as it is in Newfoundland. Interestingly however, the level of dissimilarity between ND4 diversity in Newfoundland individuals and those in other Canadian populations is unexpected. ND4 haplotype diversity in Newfoundland individuals suggests that influence from a region where haplotype H1 makes up a larger proportion and minor haplotypes (H6, H10) are present, unlike results from Ontarian and Nova Scotian samples. Thus, some connectivity between

Newfoundland's population and some other region in North America is suggested by the data; potentially from neighbouring regions not sampled in mainland Canada and/or the USA (where Canadian populations likely originated from, given the pattern of their reported expansion in North America). Fonseca and colleagues identified haplotypes H6 and H10 primarily in New York's Suffolk County (1999) and subsequent analyses of mosquitoes from the same region identified haplotype H6 throughout New Jersey in 2004 however H10 was found in much lower frequencies (Fonseca et al. 2010). While the disparity between the microsatellite and ND4 data is not formally analyzed here, this analysis will be considered in an upcoming manuscript.

Multiple reports of increased genetic diversity leading to successful invasion have been documented in this species; successful invasions are typically characterized by multiple introductions of genetically differentiated individuals which subsequently mix in their invasive range (Zielke et al. 2014; Fonseca et al. 2010). The complement is also true, in which populations of *Ae. japonicus* with lower genetic diversity do comparably worse than those associated with multiple introductions and large scale invasions in North America and Europe. For example, Zielke and colleagues (2014) demonstrated that a population of genetically similar *Ae. japonicus* in Belgium has been relatively unchanged in its range since it was established. Unexpectedly, Nova Scotian samples collected from two locations approximately 120 km apart contained only a single ND4 haplotype (H1). Similarly, microsatellite diversity indices including rarefied allelic richness of microsatellite loci were also quite low in Nova Scotia compared with the other Canadian populations (Table 3-1). Reduced genetic diversity has been suggested as a limiting factor in the invasive potential in this species (Zielke et al. 2014). While the geographic proximity of Nova Scotia to Newfoundland suggested potential migration of individuals from Nova Scotia to Newfoundland, the higher diversity detected at the ND4 and microsatellite loci in

Ontario samples may potentially provide support for a stronger link between Ontario and central Canada with Newfoundland's population. A study focused on potential populations of this species within Newfoundland and regions directly adjacent to the Island could help shed light on this more closely. This study should also look at potential populations in other regions of Nova Scotia to ensure a representative sample is included, as well as samples from more southern regions along the eastern seaboard.

In their 2015 publication, Zielke and colleagues highlighted the genetically admixed population of *Ae. j. japonicus* in the Netherlands. These authors suggest that the admixed microsatellite signatures detected in this population are indicative of multiple introductions of this species to the region. Interestingly and similar to Newfoundland's population, this population also contained the highest degree of genetic diversity at the ND4 locus out of all populations sampled (5 haplotypes in both the Dutch and Newfoundland populations). This study may reinforce the suggested process in which a heightened degree of genetic diversity is resultant from mixing of populations founded by multiple introduction events. However, given no support for multiple independent introductions to Newfoundland, our study may suggest that once mixing occurs, this diversity may be retained during range expansion. Notably, populations in Ontario, where this species has likely existed for over a decade, have seen a reduction (albeit small) in overall genetic diversity such that the observed microsatellite diversity is on par in a smaller number of Newfoundland individuals and ND4 haplotype diversity is much higher. This may indicate that while increased genetic diversity is important for the rapid spread observed in this species, it is not necessarily required for population survival in the long term. An investigation into how genetic diversity is maintained throughout the expansion of this species' range would likely shed light on the role genetic diversity plays in enabling its spread. Such a

study should include periods both during and following this species' introduction into a novel region.

Differences noted in levels of admixture between the two multilocus genotype clusters in both Canada and Europe (Fig. 3-1) could potentially reflect the difference in age between the North American and European populations of *Ae. japonicus*. While genetic studies of this species in North America began soon after its arrival, the European populations reported on using genetic techniques were not identified until 2008 (Zielke et al. 2014, 2015). The expectation, given observations from the older North American populations, is that the strong differentiation between multi-locus genotype clusters observed in STRUCTURE in European populations will likely be reduced as mixing between these groups occurs (Fonseca et al. 2010).

Unpredictably, the STRUCTURE analysis of microsatellite signatures from all Canadian and European specimens not only displays no clear population structure between populations within Canada, it also failed to parse these geographically disparate populations; instead all individuals sampled from these populations were assigned to the same two multilocus genotype clusters. The number of genetic markers used in population genetic studies is inherently linked to the resolution in which population structure can be detected, especially in studies where the focal species has undergone a very recent and widespread range expansion (Putman & Carbone 2014). Thus, the inability for the STRUCTURE analysis to parse North American and European populations is likely a reflection of the quite recent and rapid spread of this species. While a panel of 7 loci has been used extensively to characterize invasive populations of this species in North America and Europe separately, they may not necessarily be powerful enough to delineate between the two.

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4. SUMMARY

The work presented in this thesis describes analyses of two newly detected populations of mosquitoes on the Island of Newfoundland. In the first chapter, I re-affirmed the existence of *Culex pipiens* complex members on the west coast of the Island, and described a novel population of this species in St. John's, the province's capital approximately 640 km to the east. Furthermore, I have determined the taxonomic identities of these populations; both are populations of *Cx. pipiens* s.s., though while St. John's samples were comprised of a single physiological/behavioural variant (*Cx. pipiens* form *pipiens*), samples from the west coast population were a mixture of both form *pipiens* and form *molestus*. This important finding may point to potential differences in disease risk between these regions and further investigation could provide evidence for ancestral differences or connectivity between these populations. In the second chapter, I characterized the genetic diversity of Canadian populations of *Aedes japonicus* and described potential patterns of connectivity between populations of *Ae. japonicus* in Newfoundland and those in other regions of Canada and Europe. Interestingly, the genetic diversity of the Island's population was quite high; an unexpected result given Newfoundland's isolation from the rest of Canada and the assumed recent arrival of this population relative to other Canadian populations. Additionally, while no support for connectivity between Newfoundland and European populations was detected, the evidence suggests that Ontario populations are more genetically similar to populations on the Island than are those from neighboring Nova Scotia. The results of this study suggest a North American origin for the Island's recently reported population of *Ae. japonicus*. Though while no source population was

identified, the evidence suggests multiple introductions from a variety of regions in Canada and potentially in the U.S.

Efforts to monitor mosquito populations at regional scales are principally motivated by medical risks to human populations and to inform mosquito control measures focused on nuisance mosquito species. In regions that support large mosquito populations or where viral agents have a significant influence on human health, these factors provide sufficient justification to implement the often substantial and long-term expenditure required for the existence of a formal monitoring program. However, the benefits of mosquito monitoring often extend far beyond these motivations and can contribute greatly to our basic understanding of mosquito community structure and species abundance. As well, the monitoring of invasive and potential disease carrying species can help to identify how the populations and the potential health risks associated with them may change over time. This is especially important in a period in which unprecedented human-mediated connectivity and climate warming are facilitating species' access to suitable habitat in novel regions. However this information is largely non-existent in regions such as Newfoundland, in which small human populations spread over large geographic areas and the consequent low perceived impact of mosquito activity cannot provide the necessary justification for the long-term investment required to monitor mosquito populations extensively. Noticeably, however, the relatively modest efforts already undertaken on the Island have most often produced new species records including two well-known disease-vector species (the most recent expansion reported here; see also: Hustins 2006; Basset 2014, Fielden et al. 2015). Thus, while a large-scale formal monitoring program as seen in other regions is unlikely in

Newfoundland or the Atlantic region, further effort to study these organisms in a lesser capacity is justified.

In order to understand more fully the dynamics of mosquito populations on the Island, future projects must focus not only on diversity, but also on long-term population dynamics of both endemic species and any potential invasive species currently spreading in the region. Such projects would do well to include regions geographically adjacent to the Island (e.g. Labrador and the French Overseas Territory of St. Pierre/Miquelon) as well as the potentially unique geographic placement of the Island in relation to the changing habitat availabilities as species move northward with changing climates. For example, continued study of *Cx. pipiens* in Newfoundland and Labrador should include both the Island and mainland portions of the province. Additionally, differences in physiological/behavioural forms within the two populations known to exist on the Island should be further quantified and any potential connectivity between these populations investigated. Another important question surrounding these populations focuses on the disparity in predictions of habitat suitability between these two populations (see Hongoh et al. 2012). Common garden (or transplant) experiments are a tool often used to investigate intraspecific differences in habitat suitability. By moving specimens of the same species from their native environments to a common setting, responses to changing environmental pressures (e.g. fecundity, survivability, development time) can be investigated. Evidence for intraspecific differences in environmental suitability could then be examined using a genetic toolkit. Stoks and colleagues (2013) emphasized the lack of such studies linking observed differences in these responses to underlying genetic differences. In their 2016 publication, de Villemereuil and colleagues (2016) suggest that recent advances in genomics and statistical techniques can greatly amplify the ability of these studies to identify local adaptation

versus other complicating factors (e.g. plasticity). The authors emphasize that the use of genome-wide association analysis within the context of the common garden experiment panel provided by genomic tools. Such a study could also potentially identify adaptive loci (e.g. *Pgi* locus in the fritillary butterfly; Orsini et al. 2008). Notably, in their 2015 publication, Egizi and colleagues demonstrated that the assumptions surrounding evolutionary response to environmental pressures (e.g. temperature) could be violated on very rapid time scales.

Additionally, efforts to monitor mosquitoes on the Island could benefit from citizen science initiatives; such studies implement within their design a framework for gathering vital information that is adaptable in various ways by members of the general public (Cohn 2008). Citizen science efforts have famously been implemented to investigate populations of the endangered Monarch butterfly, *Danaus plexippus* L. (Cohn et al. 2008; e.g. eButterfly, www.eButterfly.com), and more recently has become an important tool for monitoring disease carrying mosquito species in North America and Europe (Maki & Cohnstaedt 2015; also see: Invasive Mosquito Project, www.citizenscience.us/imp; Mosquito Alert, www.mosquitoalert.com). Within the context of a mosquito monitoring program, efforts could be implemented through educational programming across the province, or through social media campaigns. Within the educational system, local schools would take on the project as part of a greater lesson plan, and the importance and potential outcomes of the work are worked into the lesson plan. Additionally, citizen science platforms (e.g. NlNature, www.nlnature.com) bolstered by social media, provide a potential volunteer base and could serve as a conduit in which information on how to take part in the research can be easily and effectively disseminated to those who wish to take part. In either case, emphasis must be made to simplify reporting for the public, and to disseminate data which users can interact with in a meaningful way (see

Parsons et al. 2011). Traps previously developed from easily attainable materials (Appendix III, also see Invasive Mosquito Project) could be deployed within towns and along major roadways (e.g. at rest stops and major towns along the Trans-Canada Highway, at major air and/or sea-ports) and monitored by volunteers. Collection and preservation of specimens would be demonstrated through easily accessible material either provided directly, or through an online portal. Live and/or preserved specimens could then be transported to Memorial University directly, or preserved specimens could be stockpiled and collected locally through cooperation with government departments with shipments made at the end of each season. Identifications would then be performed by specialized personnel at Memorial University. Such an effort, if successful, would provide a cost-saving approach to the collection of specimens across the province, with an additional benefit of engaging the public in scientific discourse relating directly to their region.

The research and future directions outlined in this thesis comprise an initial effort to characterize how these medically important organisms move throughout our region. Specifically, continuation of this work could aid in the development of proactive measures regarding invasive species or a potential influx of mosquito-borne illness. In general, however, efforts such as this are vital in working towards a greater understanding of a dynamic world in the face of changing climates and unprecedented human-mediated connectivity.

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5. APPENDICES

Appendix I

This appendix contains the publication titled “The mosquito community of the Avalon Peninsula, Newfoundland and Labrador” submitted to the Journal of Acadian Entomological Society (citation follows). This publication summarizes the contemporary mosquito community across the Avalon Peninsula of Insular Newfoundland and includes numerous new records for the Island as well as a number of extirpations or suspected erroneous records from historical collections. This publication makes up a small yet significant portion of my work as an MSc student; I was involved in all aspects of its development, including specimen collection and identification, as well as the writing and editing of the manuscript. The outcomes reported in this manuscript are not necessarily pertinent to the conclusions made in the main body of my thesis; they do however provide the reader with a contemporary understanding of past and present work in the field of mosquito biology in Newfoundland and Labrador, including the composition of the mosquito community on the Island over time.

The mosquito community of the Avalon Peninsula, Newfoundland and Labrador

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Abstract

Mosquitoes were collected between 2010 and 2014 on the Avalon Peninsula, which is located on the south easternmost corner of the Island of Newfoundland. Collections were made using CDC light traps, larval collections (reared to adulthood), and mouth aspirating of specimens that had been attracted to humans or sentinel-rabbits. Adult females were keyed out to species. With the addition of published records (extending back beyond the 1970s), the mosquito community on the Avalon consists of approximately 25 species in six genera. Seven of these species had not been detected on the Peninsula before 2006, including two container breeders: *Aedes japonicus* Theobald 1901 (Diptera: Culicidae), which arrived from offshore), and *Culex pipiens* Linnaeus 1758 (Diptera: Culicidae), which was previously found on the west coast of the Island of Newfoundland.

Introduction

A significant interest in the mosquitoes of the Island of Newfoundland has been twice motivated by the emergence of diseases on mainland North America. With outbreaks of West Nile disease occurring in the western hemisphere, effort was made to detect the eastern North American vector, *Cx. pipiens*, on the Island of Newfoundland (Hustins 2006). A collection of mosquitoes was made and this West Nile vector was found on the west coast of the Island. However, the virus was not detected in these mosquitoes and this program has not been continued. The mosquito-borne viruses that have been identified in Newfoundland belong to the California serogroup viruses.

Clinical cases of California serogroup virus infections were reported across Canada during the 1970s and 1980s (Artsob 1990). Patients presented varying degrees of illness, similar to those caused by West Nile virus; that is, ranging from headache, fever, vomiting and fatigue, to the neurological conditions of meningitis and encephalitis, and even to death in rare cases (Artsob 1983; Embil et al. 1985). In response to this public health concern, researchers in Newfoundland collected mosquitoes and detected two California serogroup viruses: snowshoe hare virus and Jamestown Canyon virus (Mokry et al. 1984). Their work centered on mosquitoes within a 20 km radius of two Newfoundland and Labrador cities: St. John's (primarily) and Clarenville. They captured adult mosquitoes using dry-ice-baited CDC light traps and sweep nets.

The research we present in this communication is motivated by a continued interest in mosquito ecology and the previously reported presence of CSG viruses in Newfoundland.. And, we will be reporting in a separate publication the continued presence of California serogroup viruses on the Peninsula. Here though, using various collection techniques, we set out to identify

the mosquito community on the Avalon Peninsula. Our main collection site is Salmonier Nature Park, approximately 80 km southwest of St. John's. Similarly to Mokry et al. (1984), we also focused on St. John's and just outside this city's limits. We will be comparing the mosquito diversity we encountered at these sites to the historical records of mosquito diversity on the Avalon Peninsula. The outcome will be the most thorough assessment of mosquito species present for this area. We anticipate that this record will support studies of interspecific interactions of mosquitoes (importance of interspecific interactions reviewed by Juliano 2009), which will aid assessment of mosquito-borne disease risk for our region.

Methods

Collecting Sites

The main collection site for all life stages of mosquitoes was the Salmonier Nature Park (N47° 15' W53° 16'), located about 80 km southwest of St. John's, Newfoundland and Labrador, and falling within the Avalon Forest ecoregion (Damman 1983). Due to this region's excessive moisture and ribbed moraine topography it contains a unique combination of boreal forest plant species (Damman 1983). Within this small region, and represented within the 15 km² of the park, is a considerable diversity in ecological conditions resulting in small areas that are forest, bog, heath or barrens (Damman 1983). Additionally, collections of adults and/or larvae were made at various locations on the Avalon Peninsula, locations within the city limits of St. John's (N47° 34' W52° 42'), and within 20 km outside the city limits of St. John's (East White Hills N47° 56' W52° 71', Outer Cove N47° 65' W52° 67' and St. Philip's N47° 59' W52° 87').

CDC traps and mouth aspiration

From May to September, ten (2010 season) and six (2011 season) dry-ice-baited CDC miniature light traps (model# 512, John W. Hock) were hung in trees at Salmonier Nature Park. The trees chosen were close to the animal enclosures that are part of a 3.5 km interpretive boardwalk. Traps were activated for 18 hours at a time when temperatures rose above 10°C and when rainfall was anticipated to be minimal (< 3 mm), resulting in 19 trapping events for 2010 and 19 trapping events in 2011. In 2013, four traps were set along a fishing access trail known locally as Butler's Trail. This trail is located in the backcountry of Salmonier Nature Park and is accessed at a point located approximately 1 km SW of the public park entrance. The traps were activated for 18 hours at a time on 7 occasions between the beginning of June and mid-September.

In 2011, as part of a separate study, sentinel rabbits were deployed at Salmonier Nature Park. Taking advantage of this situation, a CDC light trap was deployed (one of the six deployed that year) near the rabbit enclosure, and during weekly blood sampling of the rabbits (22 eight hour events throughout the 2011 field season) mosquitoes that landed on a researcher or rabbit were hand aspirated. Hand aspiration of specimens that landed on the exposed arms and legs of researchers was the only method used in collecting adult mosquitoes at the Outer Cove (four 4 hour events: twice with one person only, once with four people, once with eight people) and St. Philip's (opportunistically) sampling locations.

Mosquito Larvae Collecting and Rearing

Mosquito larvae were collected from still pools of water within the Salmonier Nature Park public interpretation area, as well as 10 locations along Butler's Trail (for description of trail access see note above). Larvae were also collected from four permanent pools of fetid water in East White Hills. Site selection was based primarily on site accessibility, but effort was made to include a variety of potential habitat types (man-made ditches, natural tree holes and forest floor depressions).

Thirty buckets containing fetid water were placed non-systematically in residential areas within St. John's to function as egg laying traps. These buckets were observed over two summers, 2013 and 2014 for the presence of mosquito larvae (further details can be found in Fielden et al. 2015). In fetid pools and buckets, larvae were captured with a dipper and then they were placed in 'whirl-pak' bags. The bags were transported to Memorial University of Newfoundland (MUN) for rearing in breeders (catalogue # 1425, BioQuip). Larvae were given a diet of Nutrifin® fish meal ground to a fine powder and pads of sugar water (10% sugar

concentration) were placed on top of the breeders to feed the emerging adults until they were removed and identified.

During the 2014 season in the East White Hills site, mosquito larvae were specifically sought from within *Sarracenia purpurea* L. (Sarraceniaceae), the pitcher plant, in an effort to add *Wyeomia smithii* Coquillett 1901 (Diptera, Culicidae) to our collection.

Mosquito Identification

Adult mosquitoes (caught or reared in the lab) were placed in a -20°C freezer for a minimum of 20 minutes to immobilize or kill the specimens and then they were transferred to a chill table. Identification of specimens was made using “A Photographic Key to Adult Female Mosquito Species of Canada (Diptera: Culicidae)”, by Thielman and Hunter (2007), as well as keys by Darsie and Ward (2005).

Results

Collection efforts (larval dipping, adult aspirating, CDC light traps) produced a total of 3051 specimens between 2010 and 2014. A proportion of this total includes males that emerged in the breeders but were not keyed out, and females that were too damaged to be identified. Damage may have occurred either by the CDC light trap fans or in transporting the mosquitoes. The remainder of this collection contained 22 species in six genera (Table 1). Voucher specimens of each of the identified species were pinned and are stored at MUN (Science Building, room SN4113).

Surveys of the mosquito communities of Canada, up to and including 1979, have been collated by Wood et al. (1979); this includes adult and larval keys, as well as descriptions of each species. Within this time frame specific surveys of Newfoundland mosquitoes included collections by Freeman (1952), carried out in Gander which is located in central Newfoundland, and Harmon Field which is located near the west coast of the Island; the Vockeroth (1954) collection obtained on the Great Northern Peninsula including St. Anthony; and the collection carried out by Pickavance et al. (1970) which took place on the Avalon Peninsula. Each of these references has been documented in another study by Nielsen and Mokry (1982) with the addition of efforts by The Research Unit of Vector Pathology (RUVF) (MUN). Species that were caught and identified on the Avalon Peninsula from these sources have been listed under two headings in Table 1; Wood et al., 1979 and Nielsen and Mokry, 1982. Mokry was involved in a subsequent three-year survey (1980-1983) of mosquitoes within a 20 km radius of St. John's; however, the focus was directed to species that could be potential vectors of California serogroup viruses in the attempt to determine the most eastern point in Canada for that group of viruses (Mokry et al 1984). Also included in this Table is the more recent survey by Sarah Hustins

(2006), which spanned the province, but we have included only those specimens that she collected on the Avalon Peninsula.

Discussion

Within our collections we identified 22 species in six genera (see Table 1). Seven of these species had not been detected on the Peninsula before. Five of these have possibly expanded their distribution from central or western Newfoundland: *Anopheles earlei* Vargas 1943 (Diptera, Culicidae), *Culex pipiens*, *Aedes hexodontus* Dyar 1916 (Diptera, Culicidae), *Aedes implicatus* Vockeroth 1954, and *Aedes pionips* Dyar 1919. A sixth species, *Ae. japonicus japonicus*, is likely to have arrived recently (Fielden et al. 2015), and the source of this invasion is currently unknown, but under investigation. The two new container breeders, *Ae. japonicus japonicus* and *Cx. pipiens*, are well known vectors of human disease. The association of these populations of mosquitoes with the Island's largest city is of some concern. The final new species, *Ae. aurifer* Coquillett 1903, has not been detected elsewhere in our province. Jamestown Canyon virus was isolated from this species in a 10-year study in Connecticut (Andreadis et al. 2008). They are known to bite humans and can be a nuisance when one is near their boggy habitat (Burkett-Cadena 2013). Its occurrence on the Avalon is an expansion northeast in its documented distribution (northeastern United States and the most southern portion of New Brunswick, Canada; Darsie and Ward 2005). There are some reports of rare specimens collected along the St. Lawrence River, Quebec (Wood et al. 1979). This species' known habitat includes bogs and flooded areas, such that it is not unexpected in our region.

There are four species that have been collected in previous studies that were absent in our collections. Three of these species (*Ae. communis* De Greer 1776, *Ae. excrucians* Walker 1856, *Ae. intrudens* Dyar 1919) prefer wooded pools for larval development, and perhaps we did not sample these habitats sufficiently. The fourth species, *Culiseta minnesotae* Barr 1957 (Diptera, Culicidae), has only been collected by Hustins (2006). This species was collected on both sides

of our Island, but was more abundant in the west. Perhaps this newly established species has retreated towards the west, but further study is needed. Two species that were documented in the earliest studies, but were not collected in the last three investigations are *Ae. sticticus* Meigen 1839 and *Ae. fitchii* Felt and Young 1904. The first of these species, *Ae. sticticus*, is known to lay eggs that can lay dormant for more than five years (Wood et al. 1979). Thus it was possible, though unlikely, that environmental conditions were not conducive to emergence during our study. The second species, *Ae. fitchii*, was last collected on the Avalon before 1970 (Pickavance et al. 1970). Wood et al. (1979) warn that this species has been confused with *Ae. stimulans* Walker 1848 in northern Canada, and *Ae. stimulans* was abundant in our study. It seems likely that *Ae. fitchii*, if ever present, is now absent on the Avalon.

In conclusion, the community of mosquitoes on the Avalon Peninsula contains a minimum of 22 species in six genera. Three species (*Ae. communis*, *Ae. excrucians*, *Ae. intrudens*) are likely to be present, but were missed in our collecting efforts. Three species (*Ae. fitchii*, *Cs. minnesotae*, *Ae. sticticus*) have possibly become extirpated from the Peninsula. Of the total 25 species that make up this community, there have been seven species that have moved onto the Peninsula in the last five years, and three species that have possibly moved off.

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Table 1. Mosquito species that have been collected on the Avalon Peninsula of Newfoundland.						
Genus	Species	1979 Wood et al.	1982 Nielsen and Mokry	1980-1983 Mokry et al. (1984)	2004- 2005 Hustins (2006)	2010-2014
<i>Anopheles</i>	<i>earlei</i> Felt and Young 1904 (Diptera, Culicidae)					✓
<i>Aedes</i>	<i>abserratus</i>	✓	✓	✓	✓	✓
<i>Aedes</i>	<i>aurifer</i>					✓
<i>Aedes</i>	<i>canadensis</i> <i>canadensis</i> * Theobald 1901	✓	✓	✓	✓	✓
<i>Aedes</i>	<i>cantator</i> Coquillett 1903		✓	✓	✓	✓
<i>Aedes</i>	<i>cinereus</i> Meigen 1818		✓	✓	✓	✓
<i>Aedes</i>	<i>communis</i>		✓	✓	✓	
<i>Aedes</i>	<i>decticus</i> Howard, Dyar, and Knab 1917		✓	✓	✓	✓
<i>Aedes</i>	<i>diantaeus</i> Howard, Dyar, and Knab 1913		✓	✓	✓	✓
<i>Aedes</i>	<i>excrucians</i>	✓	✓	✓	✓	
<i>Aedes</i>	<i>fitchii</i>	✓	✓			
<i>Aedes</i>	<i>hexodontus</i>					✓
<i>Aedes</i>	<i>implicatus</i>					✓
<i>Aedes</i>	<i>intrudens</i>	✓	✓	✓	✓	
<i>Aedes</i>	<i>japonicus</i> <i>japonicus</i>					✓†
<i>Aedes</i>	<i>nigripes</i>					✓

Appendix II

This appendix contains the publication titled “The Seasonal Timing of Snowshoe Hare Virus Transmission on the Island of Newfoundland, Canada.” submitted to the Journal of Medical Entomology (citation follows). This publication summarizes work carried out along the Avalon Peninsula of Insular Newfoundland in which the seasonality of Snowshoe Hare virus is described. This publication includes a description of the seroconversion of mammalian hosts in addition to detection of this virus in infected mosquitoes. While this publication was not directly impact the main outcomes of the work reported in this thesis, it does provide a reference to one of a number of arboviral pathogen currently being circulated on the Island. In this sense, the outcomes reported in this manuscript provide the reader with a contemporary understanding of past and present work in the field of mosquito biology in Newfoundland and Labrador, including the composition of the mosquito community on the Island over time.

**The Seasonal Timing of Snowshoe Hare Virus Transmission on the Island of
Newfoundland, Canada**

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H.G., Drebot, M.A., Chapman, T.W. (2017) The Seasonal Timing of Snowshoe Hare Virus
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10.1093/jme/tjw219.

Abstract

Arthropod-borne diseases negatively affect humans worldwide. Understanding the biology of the arthropod vectors and the pathogens they harbor are moving targets as a result of climate change, ecosystem degradation, species introductions, and increased human travel. Viruses within the California serogroup of the genus *Orthobunyavirus* (family Bunyaviridae) are among the mosquito-borne viruses of concern due to their zoonotic potential. Two of these, snowshoe hare virus (SSHV) and Jamestown Canyon virus (JCV), were shown, using a combination of serology and virus isolations, to circulate on the Island of Newfoundland, Canada, in the 1980s. More recently, serological analysis demonstrated that these two viruses continue to circulate on the Island in several domesticated and wild animals. Here, we detected the seroconversion to SSHV in wild snowshoe hares and in a single sentinel rabbit. The seroconversion in the sentinel rabbit occurred in early August (2011), which corresponded to the weeks of peak mosquito collections and the timing of the detection of SSHV in suspected mosquito vectors. A portion of the SSHV S segment sequence was generated from mosquito pools collected at sites near the sentinel rabbits and phylogenetically analyzed using the Neighbour-joining method with other available California serogroup virus sequences. This analysis validated the SSHV identification but showed that the Newfoundland sequence fell outside the other SSHV sequences available, which originated from the USA between 1959 and 2005.

Keywords: California Serogroup, Culicidae, snowshoe hare virus, sentinel rabbits

Introduction

Arthropod-borne diseases affect people worldwide, and many are currently of concern as emerging diseases. The main arthropod vectors are ticks, fleas and mosquitoes, which transmit viruses, bacteria, and single-celled eukaryotic parasites to human hosts. Some of the most important arthropod-borne diseases include malaria, dengue fever, West Nile virus encephalitis, Zika virus associated microcephaly and Lyme disease. Climate change, ecosystem degradation, and accelerated long distance travel have made arthropod-borne diseases an ongoing and changing concern for human society.

An important group of mosquito-borne viruses in North America fall into the California serogroup (CSG) of the genus *Orthobunyavirus*, family Bunyaviridae (Calisher 1996). These viruses possess a negative-sense, single-stranded RNA genome, consisting of three segments (Guo et al. 2012). The CSG includes California encephalitis virus (CEV), La Crosse virus (LACV), Jamestown Canyon virus (JCV), and snowshoe hare virus (SSHV), among others. These viruses are found in different animal reservoirs but are occasionally transmitted from their natural hosts to humans through a variety of mosquito vectors. Although most exposures result in mild or asymptomatic infections more severe disease such as encephalitis caused by CEV, LACV and SSHV is also observed (Calisher 1994). LACV is considered the most pathogenic of these viruses and is genetically very similar to SSHV. Clinical cases of CSG virus infections were reported across Canada during the 1970s and 1980s (Artsob 1990). Patients presented varying degrees of illness with a range of symptoms similar to those of WNV infections, from headache, fever, vomiting and fatigue, to the neurological conditions of meningitis and encephalitis, and even death in rare cases (Artsob 1983; Embil et al. 1985). More recently, sera sampled from residents of Manitoba and other provinces in Canada revealed the continued

presence of SSHV and JCV antibody in humans in Canada and documented cases of mild and neurological illness were identified (Makowski et al. 2009; Meier-Stephenson et al. 2007; Drebot 2015). It is noteworthy that some of these sera were originally submitted for testing for WNV infection based on patient symptoms, proved to be negative for WNV antibody, but were positive for SSHV or JCV IgM and neutralizing antibody.

The primary hosts for SSHV are small mammals including snowshoe hares (*Lepus americanus* Erxleben) and squirrels (*Sciurus* spp.), whereas larger mammals such as white-tailed deer (*Odocoileus virginianus* Zimmermann) and moose (*Alces alces* L.) are important hosts for JCV (Grimstad 1989). Thirty-nine species of mosquitoes have been found to be carriers of JCV in the United States (Andreadis et al. 2008), and of these 12 have been found to also carry SSHV in Canada (Artsob 1983). Within the *Aedes* genus, the persistence of these viruses is accomplished in part by transovarial transmission from infected adult females to the developing eggs and those that are laid in the fall of the year will emerge in spring as viremic larvae that then become viremic adults (Rosen 1987). Replication of either of these viruses takes place in the vertebrate hosts, such that uninfected mosquitoes can take a viremic blood meal from the reservoir and continue the cycle. In non-reservoir species that have shown evidence of infection by SSHV and JCV, such as humans, the level of viremia appears to be low, due to reduced replication, and they are considered incidental or dead-end hosts (Artsob et al. 2006). This reduced replication in incidental hosts is one possible reason that infected individuals may not always present symptoms. The viremia in humans is also too low to infect mosquitoes as part of the transmission cycle. Therefore, humans are not good amplifying hosts for these viruses but the symptomatic infections that do occur in humans are of concern for public health.

The occurrence of CSG viruses on the mainland of Canada in the 1970s and 1980s motivated arbovirus research on the Island of Newfoundland to determine the eastern extent of distribution in North America, which provided evidence of SSHV and JCV circulation (Mokry et al. 1984). Mosquitoes were captured, identified, and screened for SSHV and JCV. Mosquitoes of 11 species were detected and investigated, and SSHV was found in *Ae. canadensis* (Theobald), and JCV was detected in a sample comprising a mixture of *Ae. abserratus* (Felt and Young) and *Ae. punctor* (Kirby). This study also reported detection of antibodies to both viruses in human and horse sera and SSHV antibodies in snowshoe hare sera. A more recent study found that snowshoe hares had a high rate of seropositivity for SSHV, mink sera contained antibodies to SSHV and JCV, and cows, horses and sheep had high rates of seropositivity for JCV (Goff et al. 2012). Our study was designed to look for the circulation of SSHV using a combination of screening for the presence of the virus in captured mosquitoes and testing sentinel animals for evidence of infection and seroconversion.

Materials and Methods

Ethics Statement

This work was carried out under the guidelines specified by the Canadian Council on Animal Care with approved protocols 13-03-AL, 12-03-AL, 11-03-AL, and 10-02-AL from the Memorial University Institutional Animal Care Committee, and Memorial University biosafety permit S-103. Work in Salmonier Nature Park was carried out with permission from Park Management.

Study Sites

The main field site for collecting mosquitoes of all life stages, wild snowshoe hare blood samples (2010), and New Zealand white rabbit (*Oryctolagus cuniculus*) blood samples (2011), was the Salmonier Nature Park (SNP) (N47°15', W53°16'). This park is approximately 15 km², is located about 80 km southwest of St. John's, Newfoundland and Labrador, and falls within the Avalon Forest ecoregion (Damman 1983). Work in SNP was conducted within the captive animal display area, a naturally forested area with a stream and several ponds. Mosquitoes were also collected at various locations on the Avalon Peninsula, which include St. John's (N47° 34' W052° 42'), East White Hills (N47° 56' W52° 71'), Outer Cove (N47° 65' W52° 67') and St. Philip's (N47° 65' W52° 67'), using mouth aspirators.

Mosquito Collection and Identification

In 2011, six dry-ice-baited CDC miniature light traps (model# 512, John W. Hock Company, Gainesville, FL) were hung in trees from May through September. The trap locations were in close proximity to the sentinel rabbit enclosure (see below) within SNP public interpretation area (animal enclosures that can be viewed by the public along a 3 km trail). Heights of the traps ranged from 1.3 m to 1.4 m above the forest floor. The traps were activated

when temperatures rose above 10°C and when rainfall was expected to be minimal (< 3.0 mm a day). Each was deployed for approximately 18 hours per day; they were set before dusk and retrieved after dawn the next day. Traps were battery powered [Power Sonic model# PS-6100 F1 (6V 12AH) or Sigma SP6-20 (6V 20AH/NB)]; and had two-litre thermoses suspended from above the CDC light trap, filled with 0.70 kg of dry ice pellets. Traps were deployed 12 times over the field season totaling 1296 hours of trapping efforts (12 events X 6 traps X 18 hours/deployment). Mosquitoes were opportunistically sampled using hand aspirators, but detailed records of these collecting efforts were minor and careful records were not kept.

Mosquito larvae were collected from still pools of water within the SNP public interpretation area. Larvae were also collected from permanent pools of fetid water in East White Hills, located on the outskirts of the city of St. John's. A 250 mL dipper was used to capture larvae. Larvae were then placed in bags and transported to the laboratory at Memorial University of Newfoundland (MUN) for rearing in breeders (catalogue # 1425, BioQuip, Compton, CA). Over the field season eighteen larval collecting trips were conducted. No subsequent analyses (identification to species or snowshoe hare viral detection) were conducted using larvae.

Adult mosquitoes were transported to MUN, or removed from breeders once they eclosed, placed in a -20°C freezer for a minimum of 20 minutes to immobilize or kill the specimens and then transferred to a chilled table. Identification of specimens was made using standard taxonomic keys (Thielman and Hunter 2007, Darsie and Ward 2005). During 2011 field season, 997 mosquitoes were collected; a subset of 685 mosquitoes was sent to the National Microbiology Laboratory (NML) for virus analysis after being grouped into 129 pools, in vials labeled with the collection week, identified species, and the site location. The remaining specimens were retained as vouchers at Memorial University of Newfoundland.

DNA Barcoding of Mosquito Species

DNA sequencing of the mitochondrial Cytochrome C Oxidase subunit I (COI) gene was used to further support initial morphological identifications of mosquitoes (Cywinska et al. 2006). A 710 base pair (bp) region was targeted using the primers LCO1490 (5'-ggtaacaaatcataaagatattgg-3') and HCO2198 (5'-taaacttcagggtgacaaaaaatca-3') (Folmer et al. 1994). One or two mosquito legs were minced per specimen (typically, one female per putative species was barcoded) in 1.5 mL centrifuge tubes. DNA extractions followed the "Mammalian Tissue" protocol from the GenElute – Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich). Amplifications were carried out using a Mastercycler EPGradient. The thermal cycler settings were as follows: 2 minutes at 92°C, then 35 cycles of 30 seconds at 92°C, 45 seconds at 42.6°C, and 45 seconds at 72°C. PCR reactions were conducted in 15 µL total volumes: 7.5 µL GoTaq Colorless Master Mix (Promega), 0.6 µL of each primer (15 µM concentration of stocks) 1.0 µL of template DNA (concentrations were not measured) and 5.3 µL of DNase free water. PCR products were purified using QIAquick PCR Purification Kit (Qiagen) as per the manufacturer's protocol. The purified product was sequenced with the forward and reverse primers at the Centre for Applied Genomics DNA Sequencing Facility (Toronto, Ontario). Consensus sequences were arrived at by comparing the bi-directional sequencing results and then used for BLAST searches of the GenBank database (Altschul et al. 1997) to identify similar sequences. The nucleotide sequences have been deposited in GenBank and the assigned accession numbers can be found in Table 1.

Snowshoe Hare Virus RNA Identification in Mosquitoes

Real-time reverse transcriptase PCR (rRT-PCR) was used to identify viral nucleic acid in mosquitoes. The mosquitoes collected in 2011 were screened using primers that target CSG

viruses: CalF5 (5'-CCTAAATTTGGAGAGTGGCAGGTGGA-3') and CalR11 (5'-TTGCTGTCCCCTACCACCCACC-3'); CalgroupF (5'-GCAAATGGATTTGATCCTGATGCAG-3') and CalgroupR (5'-TTGTTTCCTGTTTGCTGGAAAATGAT-3'; Lambert and Lanciotti 2009); BCS82C (5'-ATGACTGAGTTGGAGTTTCATGATGTTCGC-3') and BCS332V (5'-TGTTTCCTGTTGCCAGGAAAAT-3'; Huang et al. 2001). The rRT-PCR reactions were carried out with reverse transcription at 50°C for 30 minutes followed by denaturation at 95°C for 15 minutes and 40 cycles of 94°C for 1 minute, 45°C for 1 minute and 72°C for 1 minute, with a final incubation at 72°C for 10 minutes. A portion of the amplified viral genome sequence, representing nucleotides 323-901 of the S segment, was sequenced to confirm identification of the virus and to characterize its relationship to other sequences. Phylogenetic analysis was performed using MEGA v.6.06 (Tamura et al. 2013). The nucleotide sequence has been deposited in the GenBank database (note to reviewer, GenBank accession number is pending).

Animal Sentinels of Virus Circulation

During the 2010 field season, 22 snowshoe hares were captured in live traps at SNP and placed in one of two chain-linked fence enclosures. Enclosure 1 was approximately 28 m by 4.5 m and divided into five pens that housed two hares each. Enclosure 2 was one large area, irregular in shape (approximately 60 m long on one side, 45 m long on the opposite side, and each of the remaining two sides approximately 30 m), and housed eight hares. Five of the hares survived until the end of summer and contributed the most extensive blood sampling. We experienced three deaths. Two hares were casualties of a hurricane, while the third hare was taken by a great horned owl.

During the 2011 field season, 15 two-month old New Zealand white rabbits (were housed in custom outdoor enclosures at SNP. Two or three rabbits from the same litter were grouped together in the same pen. The exception was one rabbit that was placed in a pen by itself because it was suspected to be a male. However, a necropsy completed at the end of the season indicated that this individual was also female. The wire hutches were approximately 1 m wide x 1 m deep x 0.61 m high, complete with food and water trays, with a 1 m wide x 0.61 m deep x 0.61 m high plywood box attached in which they could take shelter. These hutch units allowed free access to the outdoors as well as protection from unfavourable weather conditions while allowing exposure of the rabbits to mosquitoes in the area. A wire fence enclosure of 14 m x 12 m was built around the hutches as a secondary precaution to prevent rabbits escaping. Complete virology, pathology, microbiology, and parasitology tests were completed by the supplier and showed negative results. Upon completion of the field season, the rabbits were euthanized by injection of a lethal dose of barbiturates and subjected to full necropsies. Histopathology was performed to look for any abnormalities in the brain, liver, kidney, skin, lung, spleen, striated muscle, and heart.

Blood Sampling and Testing (hare and lab rabbits)

For both snowshoe hares and laboratory rabbits, blood samples were taken from the marginal vein of the ear after the skin surfaces were sterilized with alcohol,. Sampling of the snowshoe hares was attempted every two weeks, but not all hares could be caught on each attempt. Prior to transfer to SNP on 3 June, 2011, a blood sample was taken from each of the fifteen New Zealand white rabbits and further blood samples were then collected weekly. All blood samples were centrifuged at 2500 rpm for 10 minutes to separate the serum, and 0.5-mL serum aliquots were transferred to clean tubes. Sera and cell pellets were stored at -70°C and

shipped to the National Microbiology Laboratory (Winnipeg, Manitoba) for serologic and virologic assays.

An enzyme-linked immunosorbent assay (ELISA) was used to screen the snowshoe hare sera for IgG to SSHV (Johnson et al. 2000). Positive samples were then subjected to the plaque reduction neutralization test (PRNT) (Beaty et al. 1989) for additional confirmation of presence of SSHV antibody. The titre was conducted at a 1:20 dilution. The New Zealand white rabbit sera were subjected directly to the PRNT.

Results

Mosquito Collection and Virus Screening

Collection efforts yielded 997 specimens. From this sample, fifteen mosquito species were identified (see Table 1). Four samples could only be identified to genus: 2 *Aedes spp.* (separated into banded legs and black legs), 1 *Culex sp.* and 1 *Culiseta sp.* (see Table 1). 129 pools of mosquitoes of the same species or groups of species were sent to the National Microbiology Laboratory (Winnipeg, Manitoba) for testing for the presence of SSHV (see Table 1 for pool details). These mosquitoes were all collected between May 29th and September 17th in 2011. Two pools of mosquitoes, #111 and #112, that contained 17 and 26 individuals, respectively, were positive for the presence of viral RNA. Pool #111 contained only *Aedes canadensis* whereas pool #112 contained a possible mixture of individuals identified as either *Ae. punctor*, *Ae. abserratus*, or *Ae. pionips*. Both of these pools came from the Outer Cove site during the week of 21st -27th August. The collection of these SSHV carrying mosquitoes (from all locations) over the field season is presented in Figure 1.

The two positive pools were assayed by RT-PCR to attempt amplification of a portion of the S segment of the SSHV genome. The pool #111 sample produced a 579 nt sequence, whereas #112 resulted in only 200 nt of usable sequence data. The sequences were identical over their region of overlap. The longer sequence was used for a phylogenetic analysis and compared with available SSHV sequences and other CSG virus sequences from the Genbank database (Figure 2). The Newfoundland viral sequence was observed to cluster in a SSHV clade and was confirmed as belonging to this lineage of CSG viruses. The Newfoundland nucleotide sequences were somewhat divergent from the other SSHV currently available in the GenBank database,

which are from the USA and range for identification date from 1959 to 2005. However, the viral nucleocapsid amino acid sequences exhibited over 95% similarity to the other genotypes.

Serological evidence of SSHV infections

Snowshoe hare sera collected in 2010 were analyzed for the presence of antibodies to SSHV. Three of the hares were positive at their first sampling (Table 2). One hare showed seroconversion between August 10th and September 7th, while another showed seroconversion between May 22nd and June 10th (Table 2).

The New Zealand white rabbit sera were collected weekly, one showed seroconversion to SSHV Ag. That seroconversion was detected in the August 9th sample, meaning that seroconversion occurred between August 1st and August 9th. The remaining rabbits were negative over the course of the study.

Histopathology

All 15 sentinel rabbits were necropsied and samples of major organs and tissues (heart, liver, kidney, spleen, brain, striated muscle, and skin) were collected and examined. All were considered normal, with one caveat, small lesions in the livers and lungs were present in all the rabbits. However, no histological evidence of SSHV infection was apparent in the 15 rabbits; and in particular, the one rabbit that showed evidence of seroconversion.

Discussion

Mokry et al. (1984) determined that SSHV and JCV had reached the eastern edge of North America, the Island of Newfoundland. Our study confirms that SSHV is still circulating in this region more than 30 years later. We detected antibodies to this virus in snowshoe hares, seroconversion was observed in hares, and a captive sentinel rabbit seroconverted. Viral RNA was detected in one pool of *Ae. canadensis* and in one mixed pool of *Ae. pionips*, *Ae. punctor*, and *Ae. abserratus*. The timing of virus detection (late August) in these mosquito pools matches the earlier findings of Mokry et al. (1984).

During the 2010 field season we observed one hare seroconvert in response to a SSHV infection in Spring, while a second hare seroconverted in late Summer (Table 2). The timing of the second hare's seroconversion matches the timing of our detection of SSHV in the mosquitoes the following year. We experienced difficulty with consistently recapturing the hares for blood sampling, and consequently we chose to use sentinel rabbits the following year. Using laboratory rabbits ensured that we started with known immune-naïve individuals, and the ease of handling these rabbits (compared to wild hares) allowed for weekly sample collections. One of the 15 rabbits showed a clear seroconversion to anti-SSHV antibodies in its early August blood sample, two weeks prior to detection of SSHV RNA in the mosquitoes. The histopathology data for this rabbit did not reveal any evidence of pathology from the SSHV infection, an important observation when further considering the ethics of using sentinel rabbits to monitor for SSHV.

Seropositivity for both JCV and SSHV antibodies has also been documented on the Island of Newfoundland in horses and humans (Mokry et al. 1984). Horses were 18.6% positive for JCV antibodies and 2.3% positive for SSHV antibodies. The human sera were 6.5% seropositive for JCV antibodies, 1.1% seropositive to both viruses, and 0.9% seropositive for

SSHV antibodies. More recent testing of wild and domestic animals has also established seroprevalence to SSHV and JCV in cows, mink, horses, sheep and hares (Goff et al. 2012). Anti-SSHV antibodies were detected in all five groups of animals, with the highest proportion found in the hares whereas JCV antibodies were detected in all groups but the hares.

The SSHV nucleotide sequence from *Ae. canadensis* was closely related to other available SSHV sequences (Figure 2). The introduction of SSHV to Newfoundland presumably predates 1983 when evidence of circulation on the island of NFLD was documented. The Newfoundland SSHV sequence is clearly distinct from the other available viral sequences from the USA indicating genetic diversity among SSHV strains in North America. Unpublished results show that SSHV isolates from various Canadian provinces exhibit significant genetic diversity while retaining a high degree of amino acid similarity (Drebot et al, unpublished data). The further analysis of sequence data from additional portions of the virus genome from various SSHV strains collected from different geographical regions and various time frames will be required to properly understand historical movements of the virus and evolutionary changes that have taken place.

Based on this study, there is reason to believe that SSHV detected in the 1980s (Mokry et al. 1984) has persisted on the Island of Newfoundland over the subsequent decades. Our findings, and other research, support the prediction that the overwintering portion of the candidate vector species' life cycles contributes to the persistence of SSHV. This is likely due to transovarial transmission that is typical of *Aedes* species (Rosen 1987), but more research on the factors that regulate the mosquitoes' life cycle is needed. More complete characterization of the virus genome sequence and characterization of viruses from other locations in Canada would help determine if these viruses are permanently present on the Island of Newfoundland or are

regularly transferred to and from the mainland of North America. Given the potential for SSHV and other CSG viruses to cause disease and impact both public and animal health a better understanding of the dynamic components involved in transmission of these mosquito-borne viruses warrants ongoing surveillance and study.

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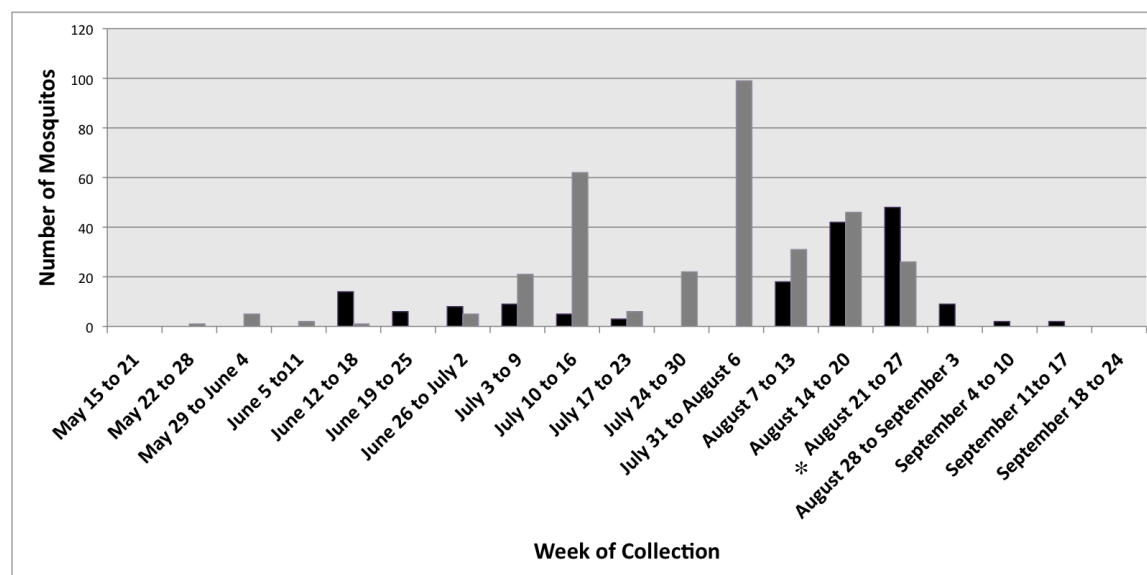


Figure 1. Temporal activity of mosquitoes carrying SSHV during the 2011 field season. The number of *Aedes canadensis* specimens for each collection period throughout 2011 are shown in black bars. Grey bars show the aggregate number of specimens for each collection period throughout 2011 of a mix of three species; *Ae. pionips*, *Ae. punctor*, or *Ae. abserratus*, ‘*’ indicates the collection period in which SSHV RNA was detected.

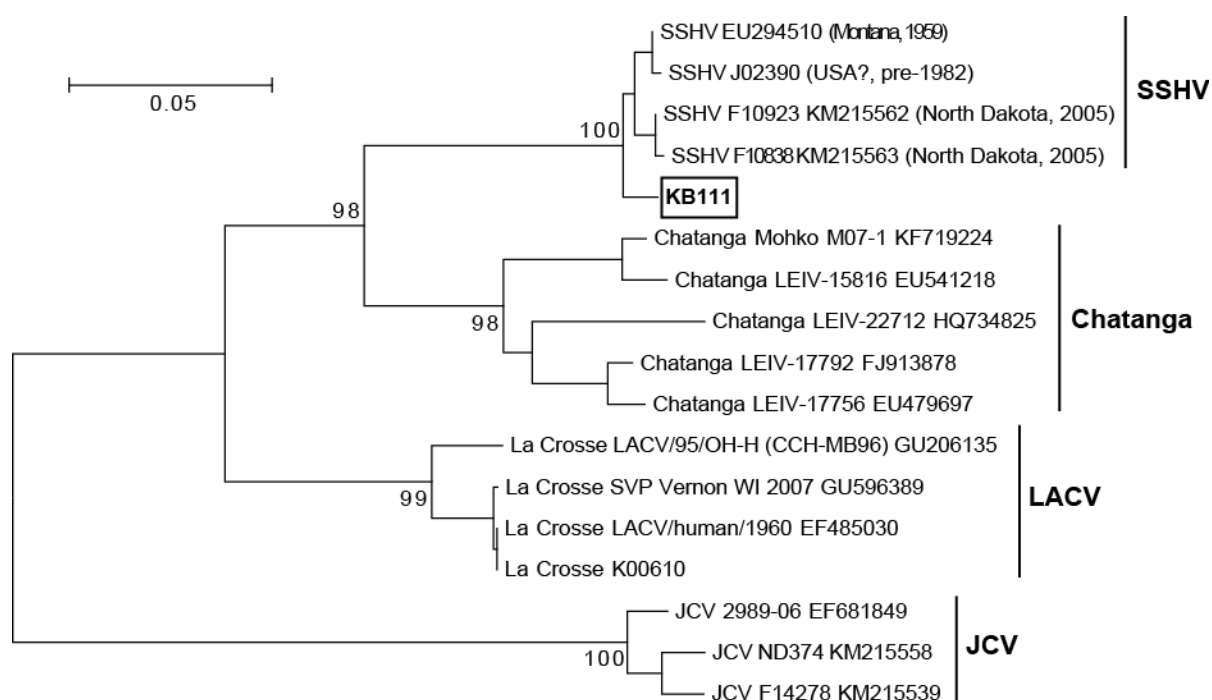


Figure 2. Phylogenetic analysis of the SSHV sequence amplified from *Aedes canadensis* in Newfoundland. The 2011 Newfoundland sequence (KB111) and the different CSG virus clades are labelled. Location and year of identification are also provided for the SSHV sequences, where possible. The evolutionary analysis was conducted using MEGA6 (Tamura et al., 2013), and using the Neighbour-Joining method (Saitou and Nei, 1987). Bootstrap values (Felsenstein, 1985) are shown as percentages based on 10000 replicates. The scale bar indicates the evolutionary distances as base substitutions per site, computed using the Maximum Composite Likelihood method (Tamura et al., 2004). All positions containing gaps and missing data were eliminated, resulting in a total of 532 positions in the final analysis. Virus designations and GenBank accession numbers are shown for all sequences.

Table 1: Mosquito species collected on the Avalon Peninsula of Newfoundland in 2011. For each species (or collection of species) the number of pools and total individuals (summed across pools) assayed for the presence of snowshoe hare virus are given. All mosquitoes were morphologically identified, and in some cases a partial sequence of CO1 provided additional confirmation (returning E scores of 0 from a BLAST search of Genbank). Voucher IDs correspond to preserved specimens housed at Memorial University of Newfoundland.

Species	Assayed for SSHV RNA, # of pools (# of total individuals)	CO1 partial sequence (Accession Number)	Voucher ID
<i>Aedes abserratus</i>	-	KF761597, KF761598	11-159, 11-227
<i>Aedes aurifer</i>	2 (2)	-	11-596
<i>Aedes canadensis</i>	21 (143) *	KF761592	11-292
<i>Aedes cantator</i>	1 (3)	KF761593, KF761594	11-105.8, 11-297
<i>Aedes cinerius</i>	5 (22)	KF761595	11-829
<i>Aedes hexodontus</i>	5 (27)	-	11-227
<i>Aedes implicatus</i>	3 (4)	-	22A
<i>Aedes pionips</i>	5 (9)	-	11-162
<i>Aedes punctor</i>	-	-	SNP-L78
<i>Aedes trivittatus</i>	1 (2)	-	-
<i>Aedes punctor/ abserratus</i>	7 (57)	-	-
<i>Aedes pionips/punctor/ abserratus</i>	8 (179) *	-	-
<i>Aedes pionips, hexodontus, punctor, abserratus</i>	3 (42)	-	-
<i>Aedes sp. (black legs)</i>	7 (37)	-	SNP-A46
<i>Aedes sp. (banded legs)</i>	3 (4)	-	DFO-117
<i>Coquillettidia perturbans</i>	15 (112)	KF761600	11-835
<i>Culex sp.</i>	1 (1)	-	-
<i>Culiseta impatiens</i>	2 (23)	KF761602	11-786
<i>Culiseta incidens</i>	1 (10)	-	-
<i>Culiseta melanura</i>	1 (1)	-	-
<i>Culiseta morsitans</i>	4 (5)	KF761603	11-836
<i>Culiseta sp.</i>	1 (3)	-	-

* indicates SSHV was detected in one pool

Table 2. Sera PRNT results for antibodies to SSHV by week of snowshoe hare blood collection, 2010.

Hare	Blood sera sample date							
	May 21	June 10	June 30	July 7	July 22	Aug 10	Sept. 7	Oct. 7
1	+	NS	NS	NS	NS	NS	NS	NS
2	NS ^a	-	NS	NS	-	-	+	NS
3	-	+	NS	+	+	+	+	-
4	NS	NS	NS	NS	NS	+	+	NS
5	+	-	NS	-	-	NS	NS	NS

^a NS means hare was not sampled

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Appendix III

This appendix contains the publication titled “*Aedes japonicus japonicus* (Diptera: Culicidae) arrives at the most easterly point in North America” published in the Canadian Entomologist (citation follows). This publication provides the first record of this species on the Island of Newfoundland and details its distribution within a sampled region of the city. Importantly, this article provides detailed collection methods for specimens used in both chapters in addition to a framework of understanding the relative distribution of these two container breeding mosquito species.

***Aedes japonicus japonicus* (Diptera: Culicidae) arrives at the most easterly point in North America**

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Abstract—*Aedes japonicus japonicus* (Theobald) (Diptera: Culicidae), the Asian bush mosquito, is a keen biter linked to the transmission to humans of a variety of diseases. It has moved significantly from its historical Asian distribution, with its arrival in North America first noted in 1998 in New York and New Jersey, United States of America. Here we report the presence of *Ae. j. japonicus* within our collections of mosquitoes in the capital city of the easternmost province in Canada: St. John's, Newfoundland and Labrador, in 2013. This observation provides further evidence of this mosquito's ability to significantly expand its geographic range, potentially affecting connectivity between subpopulations globally.

Résumé—*Aedes japonicus japonicus* (Theobald) (Diptera: Culicidae), le moustique asiatique Bush, transmet aux humains une variété de maladies via ses piqûres. L'espèce a augmenté son aire de distribution de façon significative, à partir d'une répartition historique en Asie jusqu'à son arrivée en Amérique du Nord en 1998, à New York et à New Jersey, Les états-unis d'Amérique. Nous rapportons ici la présence d'*Ae. j. japonicus* dans nos collections de moustiques dans la capitale provinciale la plus à l'est au Canada, St Jean, Terre-Neuve, en 2013. Cette observation fournit une preuve supplémentaire de la capacité de ce moustique d'élargir considérablement son aire de répartition, impactant potentiellement la connectivité entre les sous-populations à l'échelle mondiale.

Aedes japonicus japonicus (Theobald) (Diptera: Culicidae) is an aggressive biter (Kampen and Warner 2014) with one study estimating that over one third of its blood meals are taken from humans (Molaei et al. 2009). In part due to its willingness to bite humans, it is a competent vector of disease for humans in both its native (Takashima and Rosen 1989) and expanded range (Turell et al. 2001). *Aedes J. japonicus*, native to eastern Asia, was first documented in North America in 1998 when specimens were collected during a standard mosquito collection program in New York and New Jersey, United States of America (Peyton et al. 1999). Since its initial introduction to North America, *Ae. j. japonicus* has spread rapidly and is now found in over 30 states in the United States of America (Kampen and Werner 2014). In 2001, the first collections of this species were made in Canada (southern Ontario and Québec) during an annual West Nile virus (WNV) surveillance program (Thielman and Hunter 2006). The mosquito species has since spread east in Canada, through Québec and New Brunswick by 2005 and Nova Scotia by 2008 (J. Ogden, Department of Natural Resources, Nova Scotia, personal communication).

While insular Newfoundland is geographically isolated from mainland Canada, non-native insects have been known to arrive through various routes (e.g., drift migration via air currents), making direct mosquito migrations possible (Morris 1983). There is also potential for insect transfer through travel and commerce (Reiter and Sprenger 1987). Sampling of the mosquito fauna of insular Newfoundland in 2005 (Hustin 2006) and in 2011 (K.B., personal observation) did not include *Ae. j. japonicus* within their collections. However, in 2013 *Ae. j. japonicus* larvae did appear within an array of containers that were placed (with some experimental attractants, see below) in search of another container breeder, *Culex pipiens* Linnaeus (Diptera: Culicidae). The appearance of *Ae. j. japonicus* was unanticipated and,

consequently, any life history details that can be gleaned from this initial sampling are limited in scope. However, *Ae. j. japonicus* was the most abundant within our collections suggesting a rapid spread after introduction that is characteristic of this species (Kaufman and Fonseca 2014).

Twenty-seven containers (potential oviposition sites) were deployed at residential or residential-adjacent sites within the city of St. John's, Newfoundland and Labrador, Canada, non-systematically covering an area of approximately 4 km². Each container was an 11.4 L translucent white plastic bucket measuring 34 cm high with a radius of 10.3 cm. A volume of attractant solution was added to each trap sufficient to result in a depth of 5 cm (approximately 1.7 L). The attractant solutions that we describe here were part of an experiment investigating the use of an established attractant (grass: Allan et al. 2005), a potential attractant (white button mushrooms containing 1-oct-3-ol: Berendsen et al. 2013; Mathew et al. 2013) and a unique local ingredient, indeterminately aged moose feces (collected from Salmonier Nature Park, Newfoundland and Labrador). Fielden (2014) contains a full description of this experiment and outcomes. Attractant solutions that were used within the area that lured *Ae. j. japonicus* consisted of either mushrooms, moose feces, or a mixture of the two aforementioned solutions and grass clippings (collected from the St. John's area) blended with 5 L of tap water in each case. Of particular note, during the period this manuscript was in submission, we observed *Aedes japonicus* adults emerging from a bucket containing rainwater and cigarette butts. An identical comment was made by Thielman and Hunter (2006). Both notes highlight the enormous range of larval habitats these mosquitoes are capable of using. Traps were positioned in the shade beneath trees to reduce evaporation rate of the attractant solution. Also, increased shade cover is known to increase pupal productivity (Vezzani and Albicocco 2009). *Aedes j. japonicus* larvae were collected from nine of the 27 containers. Buckets were monitored weekly and when larvae were

detected they were collected and transferred to mosquito breeders (BioQuip, Rancho Dominguez, California, United States of America) that were kept in a laboratory with windows open, such that the temperature approximated exterior conditions.

Emerged adult mosquitoes were killed by freezing, pinned and identified to species using Darsie and Ward (2005). Identification was corroborated and a voucher specimen was retained by The National Identification Service of Agriculture and Agri-Food Canada (Ottawa, Ontario, Canada). Additional specimens are available in room SN-4113 at Memorial University of Newfoundland. In total, 99 *Ae. j. japonicus* adult females emerged from the collected larvae. The eclosion of larvae in the laboratory occurred between 13 August and 29 September 2013. Additionally, a single adult specimen that was naturally seeking a blood meal from a human was photographed in Outer Cove, Newfoundland and Labrador (approximately 10 km north of St. John's) on 24 September 2013 (Fig. 1). This photograph, submitted by M.E. to the citizen science website NLNature.com (which is administered by Y.F.W.), clearly shows the lyre shaped pattern of gold coloured thoracic scales, and silvery-white scale patches on the lateral thorax and abdomen indicative of this species (Darsie and Ward 2005). The detection of a new species for the province via NLNature.com is an example of how citizen science may contribute to early detection of range expansions (Catlin-Groves 2012).

Our identification of *Ae. j. japonicus* in insular Newfoundland marks an ultimate eastern boundary of the species in North America. The introduction and establishment of *Ae. j. japonicus* has the potential to alter mosquito community dynamics by outcompeting congeneric and intergeneric mosquito species (Kaufman and Fonseca 2014). Consequently, this mosquito also holds the potential to indirectly alter the viral risks of a region and, therefore, requires monitoring (Kaufman and Fonseca 2014). Kaufman and Fonseca (2014) projected a continued

but slowed expansion of *Ae. j. japonicus* into more northern regions of North America. The observations made here support a continued expansion; however, the speed of the expansion requires more substantial evaluation. A study focused on the geographic connectivity of Newfoundland and Labrador populations with populations in neighbouring and distant regions is currently underway.



Figure 1: Photograph of *Aedes japonicus japonicus* from the area of Devereaux Lane, Outer Cove, Newfoundland and Labrador, Canada during the evening of 24 September 2013. Mardon Erbland took the photograph using a Canon EOS-1D Mark IV camera equipped with a Canon MP-E 65mm f/2.8 1-5x Macro Photo lens and Canon Macro Twin Lite MT-24EX flash. A manual exposure (f/16, 1/160 sec, ISO 250) and manual flash were used. The image shows both the characteristic lyre shaped pattern of gold coloured thoracic scales, and silvery-white scale patches on the lateral thorax and abdomen indicative of this species (Darsie and Ward 2005).

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