Prevalence of California Serogroup Viruses on the Avalon Peninsula

By © P. Kate Bassett

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“Mosquito […] had asked Ear to marry him, whereupon Ear fell on the floor in uncontrollable laughter. “How much longer do you think you will live?” she asked. “You are already a skeleton.” Mosquito went away humiliated, and any time he passed her way he told Ear that he was still alive.”

- Chinua Achebe, *Things Fall Apart*

ABSTRACT

Arthropod-borne diseases negatively affect humans worldwide. Understanding the biology of the arthropod vectors and the pathogenic organisms they harbour has been a moving target as a result of climate change, degradation of ecosystems, and long distance travel. Two viruses within the California serogroup (CSV), snowshoe hare virus (SSHV) and Jamestown Canyon virus (JCV), had been identified in Newfoundland, Canada, in a study conducted from 1980-1983. Little work had been done on CSV in Newfoundland in the 30 years since. The current study was designed to determine whether these viruses still persist on the island Are these viruses still persisting on the Island; if so, which mosquito species are vectors of these viruses, and when is infection likely to occur in mammalian hosts? Evidence of infection by SSHV was found in the local snowshoe hare population and sentinel rabbits housed outdoors. The timing of observed infections was associated with peak mosquito activity. Fifteen mosquito species were collected and identified. Two pools of mosquitoes were identified as containing SSHV: one pool containing only *Aedes canadensis*, and another containing a mixed pool of *Aedes pionips, Aedes punctor*, and *Aedes abserratus*. A partial SSHV sequence was obtained from the mosquitoes and phylogenetically analyzed. Based on these findings, SSHV is currently circulating on the Island of Newfoundland.

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1 INTRODUCTION

1.1 Arthropod-borne diseases

Arthropod-borne diseases affect people worldwide. The main arthropod culprits are ticks, fleas and mosquitoes, which transmit viruses, bacteria, and single-celled eukaryotic parasites to human hosts. Well-known, headline-grabbing examples of arthropod-borne diseases are malaria, dengue fever, West Nile fever, and Lyme disease. These diseases have effects that range in severity from mild symptoms and treatable infections, to severe illness and death (Artsob, 2000). Arthropod-borne diseases often have acute impacts on their host, and evolutionary theory predicts that they should remain merciless over time by maximizing gene frequency in order to outcompete other parasites in a host, and to increase the chances of reinfecting the vector when they feed (Ewald, 1983, also see Alizon et al., 2009). Considering only the singular example of *Anopheles* mosquitoes transmitting the *Plasmodium* parasites that cause malaria (Carter et al., 2002), the number of infected people and the disease outcomes make mosquitoes the deadliest animal in the world (Vogel, 2002). Vogel (2002) advises us to know our enemy; that is, to understand the biology of the mosquito, or more generally, the disease vector. However, climate change, human degradation of ecosystems, and accelerated long distance travel have made the goal of understanding vector biology a "moving target".

Climate change contributes to this moving target by facilitating range expansion of arthropods into higher latitudes. Lyme disease in humans, caused by tick borne infections with a variety of bacteria of the *Borrelia* genus, is a clear example of this climate dependent expansion, accompanied by an increase in the active period of the tick (Brownstein et al., 2005). Travel and trade also continue to spread arthropod vectors and their associated diseases. Dengue

haemorrhagic fever (DHF), which is caused by the dengue virus and is transmitted by species of mosquitoes in the genus *Aedes*, reached epidemic levels in South East Asia following World War II (Halstead, 1980; Gubler, 1998). This arthropod-borne virus, also known as an (arbovirus), then traveled across oceans to Central and South America in cargo harbouring infected mosquitoes (Whitehorn and Farrar, 2010). By the 1960s, DHF was a significant problem in the Americas, leading to another epidemic in Cuba in 1981 (Guzmán, 1983).

The most widely distributed arbovirus in the world is West Nile virus (WNV) (Kramer et al., 2008). First detected in Uganda in 1937 (Smithburn et al., 1940), WNV has spread through Asia, Europe and into parts of the United States and Canada. The spread of this virus appears to have been greatly enhanced by human development (Crans, 2013 [a]; Savage et al., 2008). The small fetid pools of water that can accumulate in agricultural settings, around our buildings, and in our cast-off containers and other garbage, have enhanced the breeding sites for several of the mosquito vectors of this disease in and around urban communities.

1.2 Mosquito-borne viruses

In the case of mosquito-borne viruses, the viral load that is passed onto hosts during a blood meal is partially determined by the length of time that the virus has to incubate and replicate within the vector (Reiter, 1988). When environmental conditions are less than optimal for mosquito activity the mosquito may become dormant, which in turn increases the viral load that is transmitted to the host as viral replication can continue during periods of mosquito dormancy. Changing climate and range expansion can therefore be part of a positive feedback loop stimulating virus movement. As viruses are introduced into new areas with the movement of the associated vectors, they adapt to the new fauna, infecting new mosquito species that in turn can expand the host range of the pathogen (Kilpatrick, 2011). It is been suggested that

primary vectors of WNV in North America were introduced by trade and travel, *Culex pipiens* Linnaeus (Diptera: Culicidae) being one of them. Although this species appears to also play a major role in the transmission of WNV in Europe, there is little evidence that *C. pipiens* is a vector in South Africa, and for Africa as a whole, the primary vector is *Culex univittatus* Theobald (Dipter: Culicidae). Climate can also affect vector behaviour and development, presenting another challenge for anticipating arbovirus outbreaks. The vectors of WNV in North America appear to thrive in anthropogenically altered landscapes, which increases the likelihood of infection within human populations. During the process of biological transmission from vertebrate host to mosquito host, the proboscis takes up the virus with a blood meal from the infected host, the virion then binds to epithelial cells in the mid-gut of the mosquito in which replication occurs. Virions then bud-off and migrate in the hemolymph to various tissues and organs including the salivary glands where they further replicate. The virus is then transmitted from the mosquito to a new host with the coagulating fluid during a subsequent blood meal (Mullen and Durden, 2002). In cases of mosquito dormancy the biting rate decreases, however, the incubation period for virus replication within the vector increases (Reiter, 1988). Several mosquito species in the genus *Aedes,* common vectors for transmitting California serogroup viruses (CSV), rely on 'bet-hedging' to ensure survival. Some adults will lay dormant while others lay eggs, thereby staggering the developmental cycle (Reiter, 1988). However, if environmental changes have a negative impact that triggers dormancy of the whole population, the increased mosquito activity after the dormant period can result in vectors carrying an increased viral load level that could potentially lead to an epidemic, but the timing of such an event is very unpredictable.

1.3 Reservoirs

In addition to favourable environmental factors, persistence of viruses such as CSV, requires that the viruses be capable of replication in the vector host, as well as the vertebrate host, year after year. Within the *Aedes* genus, the persistence of CSV is satisfied by transovarial transmission from infected adult females to the developing eggs; eggs that are laid in the fall of the year will eclose as viremic adults in the spring (Rosen, 1987). The definitive (or primary) hosts, considered for snowshoe hare virus (SSHV), a virus within CSV, are small mammals including snowshoe hares (*Lepus americanus*) and squirrels (*Sciurus spp*.). Another CSV, Jamestown Canyon virus (JCV), includes white-tailed deer (*Odocoileus virginianus*) and moose (*Alces alces*) as the definitive vertebrate hosts (Grimstad, 1989). Replication of both of these viruses takes place in the respective hosts, such that mosquitoes can take a viremic blood meal from these vertebrate reservoirs and continue the cycle. Several other wild and domestic animal species that have shown evidence of infection by SSHV and JCV but the viremia level in these hosts appears to be too low, due to low replication, for them to be considered reservoirs (Artsob et al., 2006). This is the case with humans; they are considered incidental or dead-end hosts for CSV. When an infected mosquito feeds on human hosts or other incidental hosts they may or may not present symptoms, and because CSV do not replicate well in humans, viremia is low enough that blood meals by subsequent mosquitoes will not contain sufficient virus for transmission. Therefore, humans do not allow for the persistence of these viruses but infection is still of concern for public health.

As mentioned above, new vector species have emerged as primary transmitters of WNV with the expansion of the virus; new vector species also means a possible expansion of hosts that are exposed to the virus. This would suggest that we could expect changes in the existing

reservoirs and an expansion of incidental and/or amplifying hosts and their virulent mosquito hosts as climate changes, human travel continues, and land continues to be developed. The major mosquito-transmitted agents of highest public health concern (i.e. WNV, dengue virus, and the *Plasmodium* parasites) have not yet appeared on the Island of Newfoundland. Situated in the Northwest Atlantic Ocean, the arthropod fauna (also assumed to be true for the Insecta) of Newfoundland is the most Europeanized in North America (Biological Survey of Canada, 2012). This chimerical fauna in combination with a modern mobile human population might make this region a natural conduit for arthropod-borne disease movements between Europe and North America. Similarly, the overlap of North American and European migratory bird flyways (Olsen et al., 2006) in Newfoundland, provide such conduits for avian influenza virus movements (Wille et al., 2010). According to both published and unpublished work on mosquitoes, over 30 species have been collected and identified for the Island, which is presumably sufficiently diverse to harbour some disease agents of concern. Therefore, it is not surprising that two mosquito-borne human viral diseases have been identified in Newfoundland. The following is a summary of the mosquito and arbovirus research that has been conducted on the Island and an outline of the specific aims of this thesis to extend this work.

1.4 Mosquito and arbovirus work on the Island of Newfoundland

The mosquito-borne viruses that have been identified in Newfoundland belong to the CSV mentioned above. These represent a group of arboviruses belonging to the genus *Bunyavirus* within the family *Bunyaviridae* (Wang, et al., 2009). Humans are incidental hosts in the transmission cycle of these viruses, but two of them, La Crosse virus (LACV) and California encephalitis virus (CEV), have demonstrated severe central nervous system (CNS) infections in

the United States (Calisher, 1994). LACV is considered the most pathogenic of the CSV and is genetically very similar to SSHV. Like all CSV, both SSHV and LACV are RNA viruses, which have a high mutation rate; if SSHV mutated by recombining with LACV, the potential for increased severity of SSHV infection becomes of greater concern for public health, in particular CNS infection (Gentsch and Bishop, 1976; Holland et al., 1982; Calisher, 1994).

Clinical cases of CSV 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, 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). The detection of CSV in Ontario, Quebec and Nova Scotia in the early 1980s motivated arbovirus research on the Avalon Peninsula of Newfoundland from 1980-1983 (Mokry et al., 1984). The CSV detected in Newfoundland were the snowshoe hare virus (SSHV) and Jamestown Canyon virus (JCV). These viruses are structurally similar in that they have an enveloped virion and a genome composed of negative sensed single-stranded RNA (ssRNA) that comprises three segments: S (small), M (medium), and L (large) (Guu et al., 2012). Each of these segments codes for a different structural protein; S ssRNA codes for the N protein, which is a nucleocapsid protein that surrounds each of the three ssRNA segments; M ssRNA codes for the two external glycoproteins, Gn and Gc, that are found embedded in the viral envelope and are required for recognition of the virus in a neutralization assay or haemagglutination assay, as well as the ability to adhere to host cell membranes; L ssRNA codes for the polymerase protein which is required for replication of the genome (Elliot, 1990). Each virus has its own unique antigenicity based on at least one of the two surface glycoproteins, which enables accurate identification using an antibody neutralization assay that relies on the specificity of antibodies to

a specific virus and their ability to neutralize those virus particles (Gentsch and Bishop, 1976; Andonova and Drebot, 2008).

The objective of the Newfoundland research group in the 1980s was to establish the eastern most point of these arboviruses in North America. Their work centered on the collection of mosquitoes within a 20 km radius of St. John's, Newfoundland. They captured adult mosquitoes using dry-ice baited CDC light traps and sweep nets, and identified the viruses isolated from mosquitoes as SSHV and/or JCV. Thirty-nine species of mosquitoes have been found to be carriers of JCV in the United States (Andreadis et al., 2008). Of these 39 species, 11 also carry SSHV in Canada (Artsob, 1983). This Newfoundland-based study resulted in the isolation of virus from three carriers, *Ae. abserratus* (Felt and Young) (Diptera: Culicidae), *Ae. punctor* (Kirby) (Diptera: Culicidae), and *Ae. canadensis* (Theobald) (Diptera: Culicidae), in the 11 species investigated (Mokry et al., 1984). This same study successfully detected antibodies to both viruses in human and horse serum samples, and the detection of anti-SSHV antibodies in snowshoe hare serum samples. Although the primary mammalian hosts that act as reservoirs for SSHV and JCV have not definitively been determined, antibody titres in blood samples from snowshoe hares, squirrels and some small rodents suggest that the SSHV replicates sufficiently in these animals to contribute to the virus lifecycle, and results from moose indicate a contribution to the JCV lifecycle (Grimstad, 1989).

Some 20 years later, sera sampled from residents of Manitoba between 2004-2008 revealed the continued presence of SSHV and JCV infections in the human population in this region of Canada (Makowski et al., 2009). A portion of these sera were originally submitted for testing for WNV infection, based on patient symptoms, but proved to be negative for WNV exposure and positive for SSHV or JCV exposure, thereby showing that these viruses were still

circulating in the human population of Manitoba. Concern about WNV in Newfoundland at the time was not neglected. A survey reported by Hustins (2006) concentrated on the primary carrier of WNV in Eastern North America, *C. pipiens*. A substantial population of *C. pipiens* was detected on the west coast of the Island, however WNV was not detected. The Manitoba research then raised the concern that SSHV and JCV might also be persisting in Newfoundland.

1.5 Aims

My thesis work is focused on the surrounding area of St. John's, NL, and aims to assess mosquito diversity, to test for CSV in these mosquitoes, and thereby identify carrier species of mosquitoes in this region. These aims are designed to expand on previous studies in Newfoundland, and contribute to a baseline for future monitoring. With the confirmation that SSHV and JCV have both persisted on the mainland of Canada, based on the findings noted above (Makowski et al., 2009), I predict both of these viruses are persisting in Newfoundland. The primary mosquito vectors for CSV have been shown to involve species in the *Aedes* genus; because species of the genus *Aedes* have the ability to overwinter and maintain viable virus within, as well as their monoclonal life cycle, I predict that snowshoe hares and New Zealand white rabbits will become infected in early spring when adults eclose and again in late summer/early fall when adults are feeding prior to ovipositing.

2 MATERIALS AND METHODS

2.1 Collecting Sites

The main field site for collecting all life stages of mosquitoes, wild snowshoe hare blood samples (2010), and New Zealand white rabbit blood samples (2011), was the Salmonier Nature Park (SNP) (N47^{o} 15' W53^{o} 16'). The Park is approximately 15 km^{2} and is located about 80 km southwest of St. John's, Newfoundland and Labrador (Fig. 2.1), and falls within the Avalon Forest ecoregion (Damman, 1983). This is a small ecoregion and due to its excessive moisture and ribbed moraine topography it contains a unique combination of boreal forest plant species. However, the ecological conditions in SNP are considerably diverse, resulting in small areas that are forest, bog, heath or barrens (Damman, 1983). The Park has an interpretation centre and captive native animals on display for the public. In support of this educational initiative are staff, maintenance, animal care, and office buildings that were made available to support this research. All my work in the Park was conducted within the captive animal display area, a naturally forested area with a stream and several ponds. Animals are enclosed in this natural setting and these enclosures are connected (permitting easy access by staff and visitors) by a loop of boardwalk that is approximately three km long. Snowshoe hare are captured in live traps from the backcountry of the Park (typically not accessible to the public) by the Park staff and these specimens are routinely placed in one of two enclosures. The Park staff built special hutches and a separate enclosure for the experimental rabbits in 2011 that was out of public sight yet near the public display enclosure of the snowshoe hares. The smaller hutches enabled me to collect blood samples from the rabbits (described below).

I also collected adult mosquitoes at various locations on the Avalon Peninsula, St. John's, East White Hills, Outer Cove and St. Philip's; Hannah Munro, a fellow graduate student, also collected some during a two-week period in May 2011, near Burgeo on the southwest coast of the Island (Figure 2.1). These collections were made using hand-held aspirators (BioQuip catalogue #1135A).

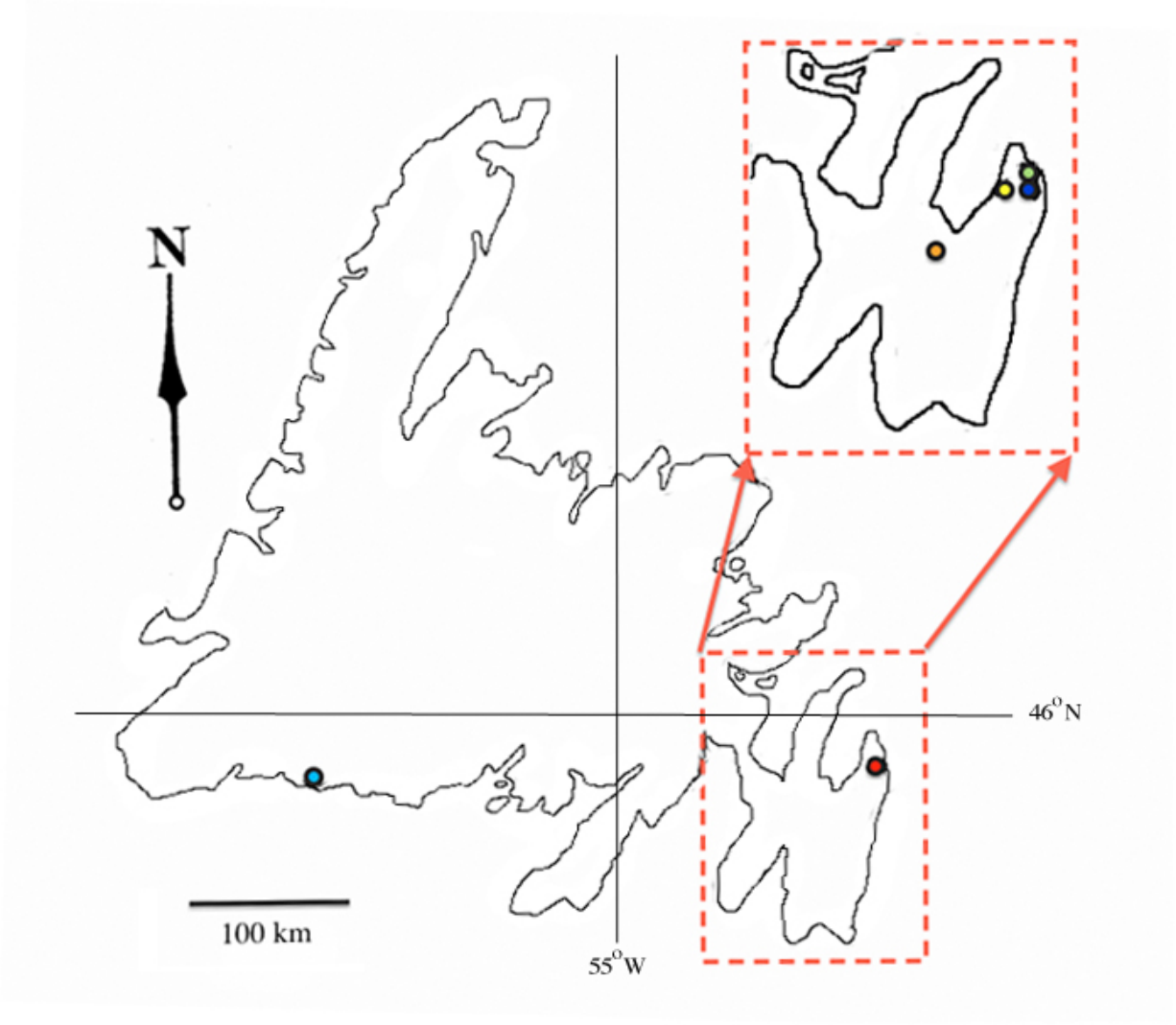


Figure 2.1 A map showing the adult mosquito collection sites on the island of Newfoundland, Canada. Mosquitoes collected at the Avalon sites were carried out by me; those collected at the Burgeo site were carried out by a fellow graduate student, Hannah Munro, during two weeks in May 2011.

2.1.1 Mosquito Collecting

From May to September of 2010, ten dry ice-baited CDC miniature light traps (model# 512, John W. Hock Company) were hung in trees at haphazardly chosen sites within SNP at three different heights, 1.52 meters (m), 1.37 m, and 1.06 m. Traps were placed within close proximity to the two snowshoe hare enclosures (Figure 2.2), other animal pens in the Park, and potential mosquito breeding grounds. According to manufacturer's guidelines, they were kept at least 9 m away from buildings (John W. Hock Company, retrieved March 2013). Traps were activated when daytime temperatures rose above 10°C, with an optimum of 21°C (Rowley and Graham, 1968), and when rainfall was minimal. The collection of adult mosquitoes during 2010 took place at SNP only, following the schedule presented in Table 2.1. This was expanded upon in 2011 to a weekly schedule (Table 2.1) and included additional sites to assess mosquito diversity at coastal locations relative to inland sites (Table 2.2): East White Hills, Outer Cove, Burgeo, St. John's, and St. Philip's. Traps were set before dusk and ran for approximately 18 hours before being 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)]; each had a two litre thermos suspended from the trap, filled with 0.70 kg dry ice pellets (purchased from Air Liquide), and I ensured that the spout was open on the lid to allow sublimation of the dry ice to CO_2 gas.

Collections made in SNP through May to September of 2011, again, used ten dry ice-baited CDC miniature light traps. However, this time the traps were set under a dense canopy of trees in an area of high moisture (as evidenced by presence of moss) and were suspended 1.37 m to 1.40 m above ground; criteria were based on correlation analyses of the 2010 field season results regarding habitat variables and the likelihood of the trap catching mosquitoes (Results did not provide outcomes that differ from the basic literature on mosquito habitat, therefore details

are included in Appendix A). The trap locations were also near the enclosure that was constructed to house eight rabbit hutches (see description below, under section 2.2 and Fig. 2.2). Hand-held aspirators (BioQuip catalogue #1135A) were employed to collect mosquitoes that were flying directly around the rabbits in order to identify the species that were potentially feeding on them for a blood meal. Site location and date were recorded for collected specimens. Collection vials were changed when the collection location changed or at the end of a collecting period; minimum 1 hour, between 10:00 AM and 3:00 PM. The changing of vials was done by placing the aspirator apparatus into a -20°C freezer for 20 minutes in order to immobilize the mosquitoes, the vial was then removed, capped, and placed back into the freezer for later identification.

Figure 2.2 SNP ground map, showing relative association of snowshoe hare enclosure sites #1 and #2 and mosquito traps (X), for 2010; 2011 mosquito traps were placed directly around the rabbit hutches (R). Two still pools within a disturbed, overgrown fen were sites of larval collection (**L**) 2011. Site #1 extends beyond the map, barely visible from the visitor's trail (highlighted by red circles).

Table 2.1 The collection schedule of adult mosquitoes and larvae during the 2010 and 2011 field seasons. Each site location indicates the weeks in which collections were made (+) during the respective years, from May to September.

2010																				
	May				June				July				August					September		
Week Number																				
Location	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38
Salmonier Nature Park					+	+		+	+	+	+	+	+	+	+	+	+			
2011																				
Salmonier Nature Park			+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
East White Hills						+	+	+	+	+	+									
Outer Cove													+	+	+	+				
St. Philip's					+			+		+					+					
Burgeo	+		+	+	+															

Table 2.2 The proximity of mosquito and larval collection sites to coastal waters, >1 km considered inland, <1km considered coastal. The distinction between inland and coastal sites was to be inclusive of species found in one habitat but not the other.

Site Location	GPS Coordinates	Proximity to St. John's	Coastal/Inland
St. John's	N47° 34' W052° 42'	----------	Inland
Salmonier Nature Park	N47^{o} 15' W53^{o} 16'	80 km southwest of St. John's	Inland
St. Philip's	N47^{o} 59' W052^{o} 87'	15 km west of St. John's	Inland
White Hills	N47^{o} 56' W52^{o} 71'	6 km north of St. John's	Coastal
Outer Cove	N47^{o} 65' W52^{o} 67'	12 km north of St. John's	Coastal
Burgeo	N47^{o} 61' W57^{o} 61'	350 km southwest of St. John's	Inland

2.1.2 Mosquito Larvae Collecting and Rearing

Mosquito larvae were collected from still pools of water within a disturbed clearing at SNP. The clearing was an overgrown fen adjacent to the site of the mosquito traps, hare enclosure number 2, and the rabbit enclosure (Fig. 2.2). A 250 mL dipper with an extendable handle was used to scoop larvae. Samples were placed in 'whirl-pak' bags and transported to the laboratory at Memorial University of Newfoundland (MUN) for rearing. Mosquito "breeders" were made out of 2 litre pop bottles to mimic commercial BioQuip mosquito breeders, (see Bioquip catalogue #1425). Each bottle was cut into three sections with the bottom section discarded, and the top section with the spout intact was inverted into the middle section, creating a funnel through the middle section; this end was fitted over a small dish that held the collected water and larvae. The open end of the middle section was covered with mesh to prevent adults from flying away. The design of the bottle allowed adult mosquitoes to eclose from the pupae in the dish and fly up through the neck of the bottle into the meshed column. Keeping them contained in this column allowed containment with airflow until the adults were collected from the breeders. The column portion of the bottle was removed once adults reached the mesh and they were transferred to a -20^{o} C freezer for a minimum of 20 minutes in order to immobilize them for identification purposes.

2.1.3 Mosquito Identification

Mosquitoes that were collected from all sites during the 2010 and 2011 field seasons were transported to MUN, placed in a -20°C freezer for 20 minutes to immobilize the specimens and then transferred to a chilled table to prevent thawing which could potentially degrade the viral RNA if present (see below). The chill table consisted of a Styrofoam box, partially filled

with dry ice and covered in aluminum foil. Identification was made using, "A Photographic Key to Adult Female Mosquito Species of Canada (Diptera: Culicidae)", by Thielman and Hunter, 2007. The specimens that were sent to the National Microbiology Laboratory (NML) for virus analysis were stored in vials according to the collection week, identified species, and the site location. Subsets of specimens were pinned and labeled as vouchers and are being stored at MUN (room number SN4113).

2.1.4 Virus Identification in Mosquitoes

The Zoonotics Laboratory at NML used Real-time Reverse-Transcriptase PCR as the diagnostic method to identify viral nucleic acid in the mosquitoes. The mosquitoes collected in 2011 were screened using primers that targeted SSHV. This method is very sensitive and has the advantage of detecting viral nucleic acid even when the viral load is low. A portion of the amplified viral genome sequence, representing part of the S segment, was sequenced to confirm identification of the virus and to characterize the relationship to other strains of SSHV (Ont 61, Yukon 80, Sask 93, and the Burgdorfer prototype isolated in Montana in 1959).

2.2 Hares and Rabbits as Sentinels of CSV Circulation

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-103-08.

Over the course of the 2010 field season 22 hares were captured in live traps by Park employees and placed in one of two chain-linked fence enclosures. Enclosure number one is approximately 28 m by 4.5 m, divided into five pens that can house two hares each. This area has

few trees and is adjacent to a grassy field, with limited shelter from potential predators. Compared to enclosure number two, these five pens are on a northeast elevation, and have a limited view from the public boardwalk. Enclosure number two is one large area, irregular in shape, approximately 60 m long on one side, 45.4 m long on the opposite side, and each of the remaining two sides is approximately 30 m. The public boardwalk runs through the middle of this enclosure, under which the hares are able to roam and take shelter.

For the 2011 field season, 15 two-month old New Zealand white rabbits were shipped on May 9 to the Animal Care Facility in the Health Science Centre in St. John's. Each of the 15 rabbits originated from one of three litters. The rabbits were all expected to be female to ensure that reproduction did not occur in the field. Two or three rabbits from the same litter were grouped together, except for one rabbit that was thought to be a male and which was placed in a pen by itself (see Table 2.3). Complete virology, pathology, microbiology, and parasitology tests were completed by the supplier prior to shipping. At the Animal Care Facility, I handled the rabbits on a regular basis in order to familiarize them with human contact; this was necessary to minimize the stress on the rabbits during weekly blood sampling in the field over the course of the season. Prior to the transfer of their transfer to SNP on June 3, each rabbit had an initial blood sample taken from the marginal vein of the ear; this was done with the aid of Bob Kelly, the Vivarium supervisor. This initial sample ensured that all serology test results were negative for antibodies to CSV before exposing the animals to any potential mosquito-borne viruses in the boreal forest habitat.

In 2011, eight wire-surrounded hutches were constructed by Park staff, such that the rabbits had approximately 1 m wide x 1 m deep x 0.61 m high enclosure, complete with food and water trays, with a 1m wide x 0.61 m deep x 0.61 m high plywood box attached in which they

could take shelter. The hutch units for the rabbits were constructed to allow them free access to the outdoors as well as protection from unfavourable weather conditions while ensuring exposure of the rabbits to mosquitoes in the area (see Appendix B for sketch). A wire fence enclosure was built around the pens as a secondary precaution against the escape of rabbits during the bleeding process and to prevent direct interaction with wildlife. This enclosure was 14 meters by 12 meters. Upon completion of the 2011 field season, the rabbits were euthanized by injection of a lethal dose of barbiturates, carried out by Dr. Laura Rogers (board certified veterinary pathologist, Animal Health Division, Department of Natural Resources). This was followed by a necropsy on each rabbit as outlined in section 2.2.3 below.

2.2.1 Blood Sampling

Blood sampling was carried out in 2010 on wild snowshoe hares, and on the New Zealand white rabbits in 2011. During the 2010 season, the snowshoe hares that were live trapped were placed in either enclosure one or two; over the season, 10 were held in enclosure one and 8 were held in enclosure two for visitor display. Sampling was attempted every two weeks, to minimize stress on the wild animals; this involved tracking and catching the hares within the enclosure. This process did not always allow for consistent sampling as hares often hid in holes. Each hare was placed in a pillowcase to prevent it from seeing its surroundings in order to calm it and one ear was extended through a hole in the pillowcase from which the blood sample was taken. During the first two weeks of sampling, a veterinary student, Kayla Collins, collected the samples based on protocol instruction by employees of the Park. Changing from hares in 2010 to New Zealand white rabbits in 2011 enabled a more consistent weekly sampling regime. The rabbits were easier to capture because they were confined to the hutches, and handling was less of a struggle due to exposure to human contact since birth.

Blood was taken from the marginal vein in the ears of the hares/rabbits after the area was sterilized with alcohol; this was done by inserting a 20G ½" needle into the vein and creating a small tear to prevent clotting. Blood was then scooped from the ear with a 1.5 mL centrifuge tube and labeled. Samples were transported back to the Animal Health Laboratory (Newfoundland and Labrador Department of Natural Resources), located on Brookfield Road, St. John's, NL, approximately 45 minutes away from the Park. At this location, the blood samples were centrifuged at 2500 rpm for 10 minutes to separate the serum from the remaining pellet of blood. Serum aliquots of 0.5 mL were transferred to clean centrifuge tubes, leaving only the pellet in the original tube; both serum and pellet were stored at -70°C until the end of the field season at which point all samples were shipped to the NML where serological diagnostics for the presence or absence of antibodies to CSV was carried out.

2.2.2 Blood Diagnostics

While costly and very time intensive, the plaque reduction neutralization test (PRNT) is considered the "Gold Standard" for serodiagnostics because of its high degree of accuracy (Andonova and Drebot, 2008). An enzyme-linked immunosorbent assay (ELISA) is often carried out first to screen large sample sizes (Johnson et al., 2000); however, false positives can occur in the case of SSH and JC viruses due to cross-reactivity. The snowshoe hare serum samples from 2010 were screened for antibodies to both viruses using ELISA, and any positive results were then subjected to the PRNT for confirmation. As a means of reducing turn-around time for the diagnostic results, the New Zealand White Rabbit sera were not tested using ELISA and instead were subjected to the PRNT to screen for antibodies to SSH virus only. The PRNT is based on the reduction of plaque forming units (PFU) that result when the antibodies in a serum sample neutralize the virus when added to a monolayer of Vero E6 cells (Andonova and Drebot, 2008).

Plaques are colourless spaces formed where cells that became infected with the virus are destroyed. If the serum sample is positive for antibodies to the particular virus being tested, then the antibodies will neutralize the virus by binding to it, thereby reducing the number of plaques that form. The number of plaques that would form when a virus is exposed to susceptible cells in the absence of antibodies is measured at 100 plaque-forming units. When serially diluted serum samples are incubated with this measure of virus and susceptible cells, a ≥ 90% reduction in plaque formation is considered a positive result, providing the quantification of the antibody titre (Weingartl et al., 2003).

2.2.3 Histopathology

The CSV can manifest in various tissues and organs in the amplifying host. Therefore, on September 28, 2011, I assisted Dr. Laura Rogers on performing necropsies on the 15 euthanized rabbits and she carried out a histopathology work up to identify if there were any abnormalities in the brain, liver, kidney, skin, lung, spleen, striated muscle, and heart.

2.3 DNA Barcoding of Mosquito Species

DNA sequencing was used to confirm initial morphological identifications of mosquitoes. DNA extraction and subsequent amplification of the conserved mitochondrial cytochrome c oxidase subunit I (COI) gene were conducted. This targeted a 710 base pair (bp) region using the primers: LCO1490: 5'-ggtcaacaaatcataaagatattgg-3' and HCO2198: 5'-taaacttcagggtgaccaaaaaatca-3' (Folmer et al., 1994). Due to the relatively recent introduction of WNV into North America, and the necessary surveillance required to monitor hotspots of potential vectors, this method of identifying mosquito species and submitting the barcode sequences has allowed for an adequate sequence database with which to compare unknown DNA

mitochondrial COI sequences (Cywinska et al., 2006). This method has a quick turnaround time for results at minimal costs, and is highly reliable.

One or two mosquito legs were minced for each specimen in a 1.5 ml centrifuge tube. To extract the DNA, the "Mammalian Tissue protocol" from the GenElute – Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich) was followed. This protocol is based on previous successful extraction preformed by Folmer and colleagues, 1994. It was not necessary to run know mosquito samples alongside of the unknowns due to the vast number of known mosquito COI sequences that are currently available in the database. Amplification was carried out using the Eppendorf Authorized Thermal Cycler, Mastercycler EPGradients. A preliminary PCR set up with three different volumes of extracted DNA (0.3 µl, 0.6 µl, 1.0 µl) determined that the best results were achieved using a volume of 1.0 µl of DNA. The annealing temperature was initially set to 55°C; however, by running a temperature gradient PCR on three samples, this protocol was found to be optimal at 42.6°C. The PCR cocktail contained: GoTaq Colorless Master Mix (Promega), 7.5 µl; primers LCO1490, 0.6 µl, and HCO2198, 0.6 µl; DNA, 1.0 µl; and nuclease-free H_2O, 5.3 µl. The thermal cycler settings were as follows: 2 minutes at 92°C, then 35 cycles of 30 seconds at 92°C, 45 seconds at 55°C (42.6°C), and 45 seconds at 72°C. The PCR products were analyzed by gel electrophoresis. The 1.5% agarose gel, made with 2.0 g agarose and 132.0 mL TBE Buffer (0.5X) (made 4 L by mixing 200 mL TBE (10X) with 3800 ml dH_2O), was loaded with 3 µl of each PCR product and 3 µl of a standard DNA ladder. Gel Red Nucleic Acid Stain was used as the DNA fluorescent dye, and was premixed with 6X loading buffer as per the manufacturer's protocol. Amplification results were determined based on band presence and intensity under a fluorescent light. Samples with positive amplification were purified using QIAquick PCR Purification Kit (Qiagen) as per the manufacturer's protocol. Elution of the PCR

product required the addition of 50 µl of DNase-free water, instead of the elution buffer (EB) noted in the protocol. This substitution was recommended by the sequencing lab so as not to interfere with the sequencing protocol. The purified PCR product of each sample 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.

3 Results

3.1 Mosquito collection

Collection efforts in 2010 yielded 504 mosquitoes, comprised of 13 species (Table 3.1). Dr. Aynsley Thielman, a certified mosquito identification specialist and researcher, confirmed the identity of *Aedes hexodontus* Dyar (Diptera: Culicidae) during a visit to MUN (May 2011). The 2010 collection contained males, which were not identified to species (keys for males are available but require specialized techniques and males are not routinely identified to species). The collection also included damaged females that could not be identified to species (CDC light traps draw females through a moving fan that can lead to damage, obscuring critical characters necessary for species identification). Voucher specimens of each of the identified species were pinned and stored at MUN (Science Building, room SN4113).

The 2011 field season yielded 997 mosquitoes, of which 839 were identifiable females. The collection comprised 15 species (Table 3.2). Twelve of the 15 species were collected at the main field site at SNP. The identities of nine of the species from 2011 were confirmed by DNA barcoding (Table 3.3).

Culex pipiens was collected by me in downtown St. John's in 2012. This is the first record of *Cx. pipiens*, a known vector of WNV, on the east coast of Newfoundland. The identity of this species was confirmed by DNA barcoding (Table 3.3).

A subset (685 specimens) of the 2011 specimens was tested for the presence of SSHV. Two pools of mosquitoes, pool 1 (111) containing 17 mosquitoes and pool 2 (112) containing 26 mosquitoes, were positive for the presence of viral RNA. The species identified as carrying the

viral RNA were *Ae. canadensis* (111) and a mixed pool (112) comprised of *Ae. punctor, Ae. abserratus,* and *Ae. pionips.* The seasonal distribution of *Ae*. *canadensis* collection, as seen in Figure 3.1, appeared in two clusters, with SSHV RNA present in the second cluster, during the week of August 21 to 27, 2011. The seasonal distribution of species in the mixed pool was scattered from the end of May to the end of August, with the presence of the SSHV RNA also during the week of August 21 to 27, 2011. Both of these collections came from the Outer Cove site; however these four mosquito species were actively flying at the SNP during this time as well as evidenced by trap collections during this time.

Table 3.1 Mosquito species collected during the 2010 field season at Salmonier Nature Park using CO_2 baited CDC miniature light mosquito traps. Specimens were identified using a photographic adult mosquito identification key by Thielman and Hunter (2007). The mixed pools were comprised of two or three species when key characteristics were missing thereby preventing specific identification. All mosquitoes were adult females in the Family Culicidae.

Species Collected in 2010	Salmonier Nature Park
	n
Aedes abserratus	45
Aedes canadensis	156
Aedes cinereus	6
Aedes diantaeus	4
Aedes hexodontus	9
Aedes implicatus	6
Aedes nigripes	19
Aedes pionips	32
Culex pipiens	1
Culiseta melanura	9
Culiseta morsitans	30
Coquillettidia perturbans	26
Mixed Pools	
Aedes abserratus or *Aedes punctor*	18
Aedes abserratus, Aedes punctor, or *Aedes pionips*	66
Total	427

Table 3.2 Mosquito species collected on the Island of Newfoundland during the 2011 field season as female adults (A) or female larvae (L) and the numbers at each location.

Collection Site / Species	Salmonier Nature Park	East White Hills	Outer Cove	St. Philip's	Burgeo	St. John's
Aedes aurifer	A(5)		A(6)			
Aedes canadensis	A(94)	L(20)	A(52)	A(2)		
Aedes cantator		L(12)				
Aedes cinereus	A(13) L(12)					
Aedes diantaeus	A(6)					
Aedes hexodontus	A(23)		A(5)			
Aedes implicatus	A(5)					
Aedes pionips	A(3) L(8)		A(1)	A(1)	A(1)	
Culex pipiens						A(4)
Culiseta impatiens			L(1)	A(1)	A(36)	A(1)
Culiseta melanura	A(8)					
Culiseta morsitans	A(6) L(1)					
Coquillettidia perturbans	A(95)		A(12)	A(5)		
Aedes spp.	23		20	43		
Culiseta spp.	2					
Culex spp.	1					
Mixed Pools						
Aedes abserratus, *Aedes punctor*	A(60) L(52)	1	A(35)			
Aedes punctor, *Aedes pionips*, *Aedes abserratus*	A(55)		A(112)			
Totals	**472**	**33**	**244**	**52**	**37**	**5**

Table 3.3 Mosquito species collected for this study and corresponding voucher codes where available. DNA fingerprinting of the mitochondrial cytochrome c oxidase subunit 1 (CO1) gene was carried out on those species in bold to confirm identification. Corresponding accession numbers from the GenBank database for the sequences obtained are given.

Genus	Species	E-Scores	Voucher	Accession #
Aedes	***abserratus***	0 0 0 0 0	1E 15Q 11-159 11-227 13Q	KF761590 KF761591 KF761597 KF761598 KF761599
Aedes	*aurifer*	-	11-285 11-585 11-596	------
Aedes	***canadensis***	0	11-292	KF761592
Aedes	***cantator***	0	11-105.8 11-297	KF761593 KF761594
Aedes	***cinereus***	0	11-829	KF761595
Aedes	***diantaeus***	0	11-168.6	KF761596
Aedes	*hexodontus*	-	11-575	------
Aedes	*implicatus*	-	----	------
Aedes	*pionips*	-	----	------
Aedes	*punctor*	-	----	------
Culex	***pipiens***	0	12-1	KF761601
Culiseta	***impatiens***	0	11-786	KF761602
Culiseta	*melanura*	-	----	------
Culiseta	***morsitans***	0	11-836	KF761603
Coquilletidia	***perturbans***	0	11-835	KF761600

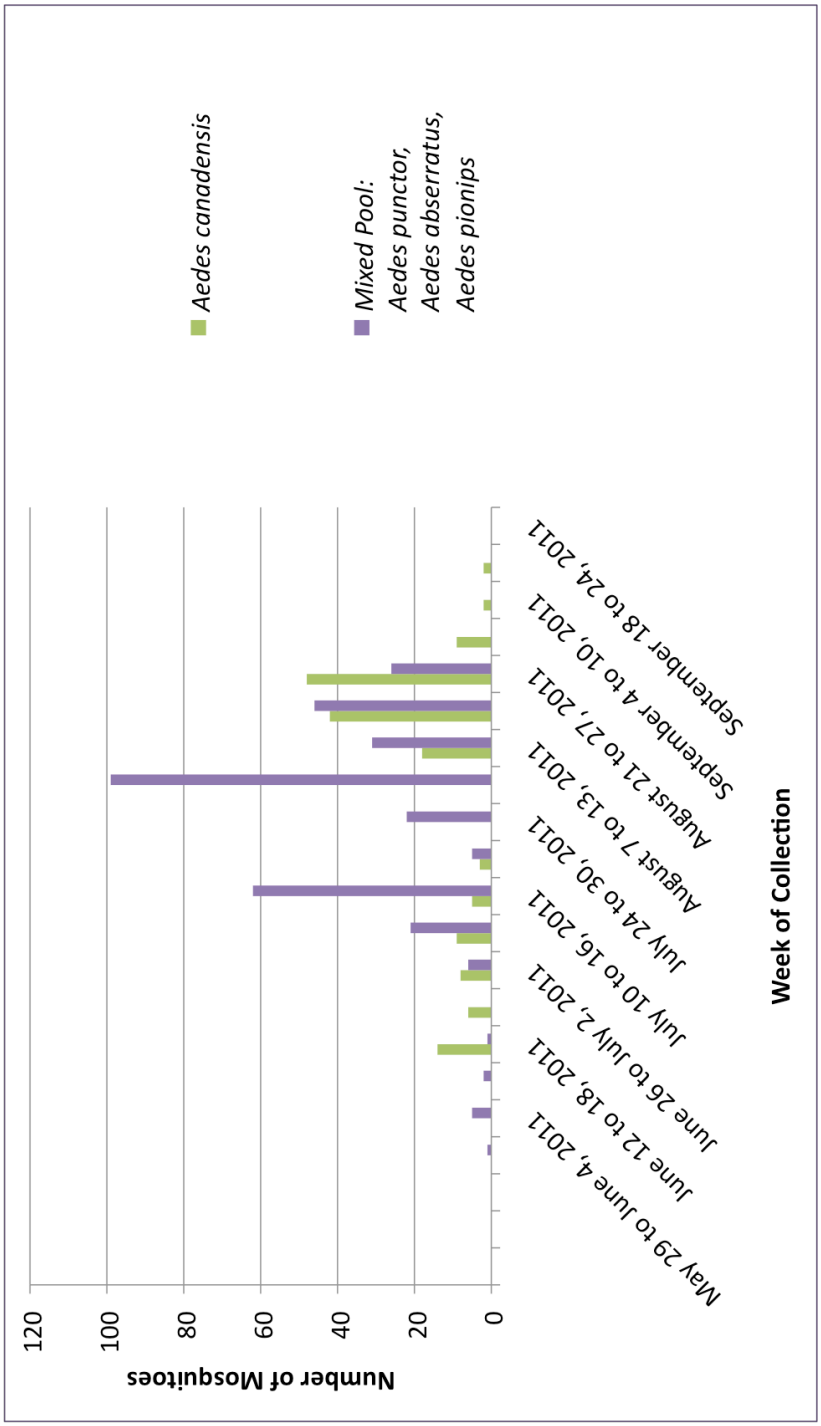


Figure 3.1 Temporal activity of mosquito species that were detected carrying SSHV during the 2011 field season. Detection of SSHV RNA occurred in both *Aedes canadensis* and the *Aedes sp*. mixed pool collected during the week of August 21 to 27, 2011. The mixed pool comprised specimens that were incompletely identified to one of three species, as indicated.

3.2 Sera sample results

Snowshoe hare blood samples collected in 2010 were analyzed by the NML, and showed that the immune system of two hares (numbers 1 and 5, see Table 3.4) were already producing antibodies in response to both SSHV and JCV infection at the time of the first collection of blood. Hare number 2 (Table 3.4) showed seroconversion for antibodies to SSHV and JCV, somewhere between July 23 and September 7 (Figure 3.2). Hare number 3 underwent seroconversion for antibodies to only SSHV between May 22 and June 10 (Table 3.4). Hare number 4 was caught on August 10, and was shown to be seropositive for SSHV when the first blood sample taken.

The blood collected from the New Zealand white rabbits were taken on a weekly basis during the 2011 field season; due to the number of blood samples submitted and workload constraints at the NML facility, the diagnostics lab was only able to test for SSHV and not JCV. Of the 15 rabbits that were placed in the field during the season, one rabbit underwent seroconversion for anti-SSHV antibodies (Table 3.5). Seroconversion in that rabbit occurred between August 1 and August 9 (Figure 3.2). The serology for the remaining rabbits was negative over the course of the study.

Table 3.4 Results for the presence of antibodies to snowshoe hare virus (+) and Jamestown Canyon virus (+) as determined from snowshoe hare serum samples from 5 hares, collected during the 2010 field season at Salmonier Nature Park.

Hare# / Week#	May	June	June	July	July	Aug	Sept	Oct
	20	23	26	27	29	32	36	40
1	++							
2*		-			-	-	++	
3**	-	+		+	+	+	+	-
4						+	+	
5	++	-		-	-			

*Note: Seroconversion between August 10 (negative result) and September 7 (positive result)

**Note: Seroconversion between May 21 (negative result) and June 10 (positive result)

Table 3.5 Results of the presence of snowshoe hare virus (+) determined from New Zealand white rabbit serum collected weekly during the 2011 field season at Salmonier Nature Park.

Week# / Rabbit#	May				June				July				Aug					Sept			
	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39
1	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-	+	+

Note: Seroconversion between August 1 (negative result) and August 9 (positive result)

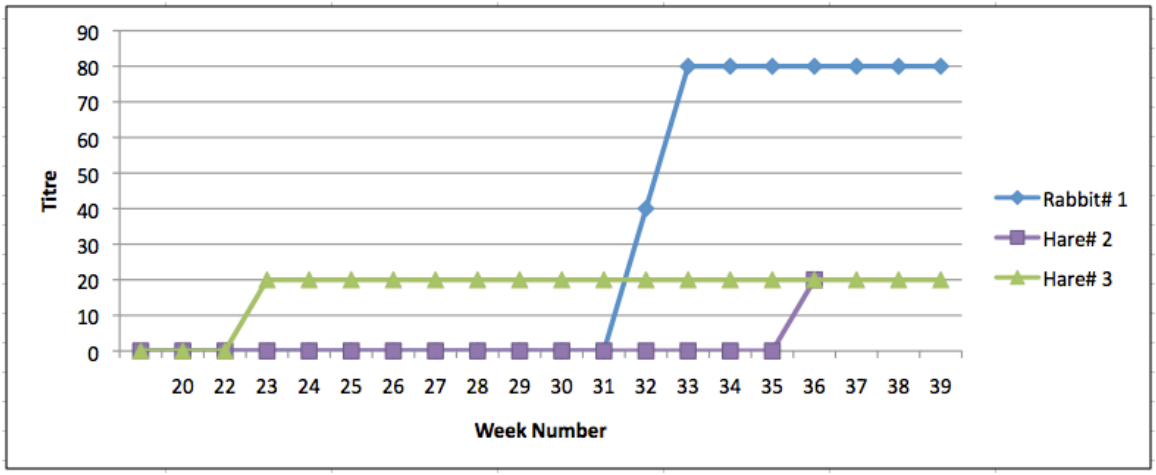
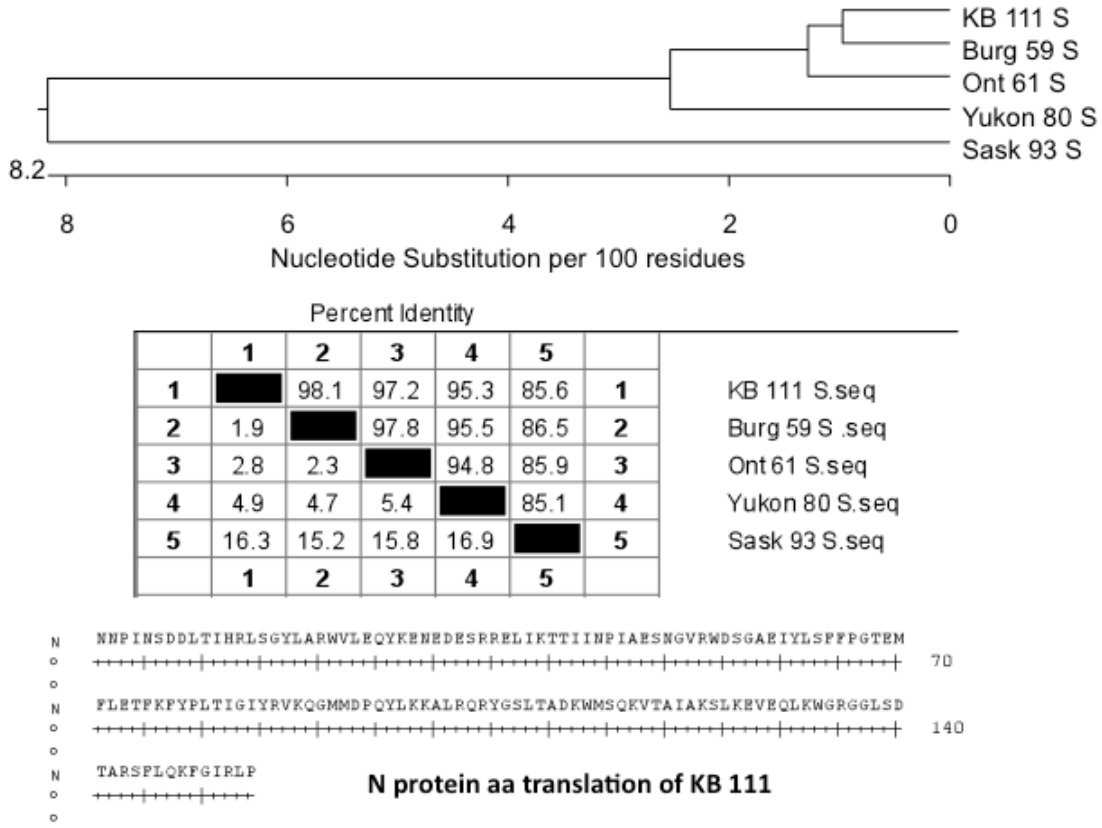


Figure 3.2 Serology results of 2010 hares, numbers 2 and 3, and 2011 rabbit number 1, showing seroconversion of antibody titres from negative to positive. Hare #2 was positive for antibodies against both SSHV and JCV, both with a titre of ≥20, whereas Hare #3 was positive for antibodies against SSHV only, with a titre ≥20; Rabbit #1 was positive for antibodies against SSHV with a titre of ≥80, but was not tested for the presence of JCV.

3.3 Snowshoe Hare virus RNA

The SSHV RNA was detected from two mosquito pools: one containing *Ae. canadensis* (RNA sequence reference #KB 111) and the other a mixed pool of one or more of *Aedes abserratus*, *Aedes pionips*, and *Aedes punctor* (RNA sequence reference #KB 112); both sets of mosquitoes were collected during the week of August 21 to 27, 2011 at the Outer Cove site. The KB 111 sample produced a 579 nucleotide sequence, whereas KB 112 produced only a 200 nucleotide sequence. The viral RNA sequences from the two samples were identical over their region of overlap. Due to the truncated sequence of KB 112, only the KB 111 sequence was used in comparison with the four well-known distinct SSHV clades: Burgdorfer prototype (Burg 59) (isolated in 1959 in Montana), Ont 61, Yukon 80, and Sask 93 (Figure 3.3; Appendix D). The S segment of the sequence (Appendix C) was used for this comparison because it is more highly conserved, particularly within the region that codes for the N protein (Elliot, 1990). The S segment of the viral RNA circulating on the Avalon Peninsula of Newfoundland was 98% conserved with the Burg 59 S, with sequence alignment from nucleotides 323 to 901 of the segment based on a BLAST search. It was also very similar to the Ont 61 and the Yukon 80 sequences, at 97% and 95.3% identity, respectively. It was most distinct from Sask 93, with 83.7% identity.



	1	2	3	4	5	
1		98.1	97.2	95.3	85.6	1
2	1.9		97.8	95.5	86.5	2
3	2.8	2.3		94.8	85.9	3
4	4.9	4.7	5.4		85.1	4
5	16.3	15.2	15.8	16.9		5
	1	2	3	4	5	

Figure 3.3 Identification and characterization of SSHV RNA in *Aedes canadensis* using RT-PCR from sample KB 111. A phylogenetic tree representing the genomic relatedness of the SSHV circulating in Newfoundland in 2011(KB 111), with those isolated in Montana, Ontario, Yukon, and Saskatchewan is shown. The percent identity contingency table is shown to illustrate the relatedness between each clade being compared. The analysis of the KB 111 sequence also includes the N protein amino acid translation.

3.4 Histopathology Results

The 15 sentinel rabbits were euthanized on September 28, 2011. Samples of major organs and tissues (heart, liver, kidney, spleen, brain, striated muscle, and skin) were removed and their general appearance and size was noted. Clinical histopathological testing was overseen by Dr. Laura Rogers. All rabbits were considered essentially normal but had small lesions in the livers and lungs. The livers of several rabbits were described as having swollen centrilobular hepatocytes with moth-eaten cytoplasms. Both of these abnormalities had undetermined etiology, though hypercellularity of the lungs was noted and the livers showed an accumulation of glycogen and/or lipids. Specific indications for rabbit 1 included the thickening of the coronary arteries, presented by microscopic lesions. Histopathological observation of rabbit 2 revealed hematopoietic precursors in the adrenal cortex of only one adrenal gland; nothing further was examined as it was presumed incidental. In addition, not all rabbits had adrenal gland examinations and thus could not be compared to the other specimens. Rabbit 3 appeared to have an infection concentrated in the interstitial myocardium suggesting infection with *Toxoplasma gondii* (Sarcosystidae), however immunohistochemistry carried out at Prairie Diagnostic Services (Saskatoon, Saskatchewan) showed negative results for this parasite. No histological evidence of SSHV was apparent in the 15 rabbits. A copy of the histopathological report is provided in Appendix D.

4 Discussion

Two California serogroup viruses, SSHV and JCV, are present on the Avalon Peninsula of Newfoundland. Through an assessment of mosquito diversity and an analysis of the diagnostics of 129 mosquito pools, SSHV RNA was detected in one pool of *Ae. canadensis* (KB 111), and one mixed pool of *Ae. pionips, Ae. punctor, and Ae. abserratus* (KB 112). Both pools were collected from the Outer Cove site during the week of August 21-27, 2011. Mosquito samples were not tested for JCV RNA because of the large number of vials submitted for testing, and the constraints this would place on turnaround time for the regular workload at NML. As well many samples had a small number of specimens per vial, thus, the virus RNA load would have been very difficult to detect. Serum samples from three snowshoe hares in the 2010 field season, in SNP were positive for antibodies against both SSHV and JCV, and two others were positive for antibodies against only SSHV. From the same area, SSHV infection was detected in one sentinel rabbit the following year (2011). Although hare/rabbit blood samples were not collected at the same site as the collection of the infected mosquitoes, the same species of mosquitoes were flying and feeding at both locations from the last week of July inclusively to the end of August. These mosquito species were observed feeding on the rabbits during the month of August, which corresponds to the timing of seroconversion of the sentinel rabbit.

The Newfoundland study by Mokry and colleagues, between 1980-1983 was prompted by clinical cases of CSV infections arising in Ontario, Quebec, and Nova Scotia at that time. The study focused on determining if these viruses had reached the eastern limit of the continent by looking for the potential mosquito vectors. Their findings showed that JCV and SSHV were circulating on the Island; JCV was isolated from a mixed pool of *Ae. punctor* and *Ae. abserratus* collected 8 km from St. John's on July 17, 1980 and SSHV was isolated twice, both from *Ae.*

canadensis, collected in Clarenville on August 18, 1982 and again on August 20, 1983. Thirty years later, my study suggests that *Ae. punctor* and *Ae. abserratus* might also be vectors of SSHV, and *Ae. pionips* may potentially be a third vector as well. The finding of *Ae. canadensis* as a vector of SSHV agrees with the results of Mokry and colleagues, as does the temporal pattern of detection of this virus. As part of a 10-year study in Connecticut, JCV was isolated from *Ae. abserratus* 16 times over 6 years, and from *Ae. canadensis* 40 times over 9 years (Andreadis et al., 2008); in that study *Ae. canadensis* was also the species with the largest spatial range. Andreadis and collegues were able to identify several other species that are capable of carrying JCV, but of particular interest are those that have also been collected here on the Island of Newfoundland: *Aedes cantator* Coquillett, *Coquillettidia perturbans* Walker, *Aedes cinereus* Meigen, *Culiseta melanura* Coquillett and *Culiseta morsitans* Theobald; (Diptera: Culicidae).

The four potential vector species identified in this study are considered snowpool mosquitoes. The eggs overwinter and develop in an aquatic habitat until they eclose as adults when the snowpool melts in early spring (Wood et al., 1979; West and Black IV, 1998). The first mosquitoes collected in May, 2011 were larvae of *Ae. pionips*. Information on the *Ae. pionips* life cycle in the literature is limited, but it is considered to be closely related to *Aedes communis* with the exception that eclosure is delayed by several weeks relative to this species (Vockeroth, 1952). JCV has been isolated from the larvae of *Ae. communis* in other studies, which suggests that *Ae. pionips* is also a likely viable vector of this California serogroup virus (Crans, 2013 [b]). *Ae. pionips* is also found in association with *Ae. canadensis*, both of which utilize disturbed lands, fens, and marshes for larval habitats (Vockeroth, 1954); this is similar to the primary habitat of both of the larval dipping sites at SNP (Fig. 2.2, Fig. 3.2) that yielded both species from the larval collection, and East White Hills that yielded *Ae. canadensis*. The primary

difference between these two sites is their proximity to the ocean with SNP inland and East White Hills coastal (Table 2.2). The 2006 study in Newfoundland, by Sarah Hustins, showed that *Ae. canadensis* larvae frequently occur in natural wetlands, followed closely by disturbed wetlands, and then by urban settings. Within these habitats, the most abundant were temporary pools. My results were similar to those of Hustins (2006) in that larvae and adults were found in and around temporary pools and disturbed wetlands at SNP and East White Hills, and adults around temporary pools and the natural wetlands at Outer Cove. This diverse range of habitats suggests this species is a widespread vector of disease. *Ae. canadensis* has a preference for feeding on small and large mammals, making it an ideal transmitter of both California serogroup viruses (Crans, 2013 [c]), which this study, in conjunction with the findings of the Mokry et al., 1984 study, has proven to be the case in Newfoundland.

All four species have a univoltine lifecycle but reproduction and deposition of eggs appear to occur periodically throughout the season. *Ae. canadensis* adults emerge in early spring, first seen in this study the week of June 12, and go on to produce a brood late in the season, eclosing as late as September 17 (Figure 3.1) (Crans, 2013 [c]). Eggs from the second brood are left to overwinter and if transovarial transmission has occurred from infected females to the developing eggs, they will continue to contribute to the persistence of CSV in Newfoundland. *Ae. canadensis* is also found in association with *Ae. punctor* and *Ae. abserratus*, as was the case at SNP and Outer Cove (Table 3.2). *Ae. punctor* is considered the most abundant species on the Avalon Peninsula, followed by *Ae. abserratus* (Mokry et al., 1984) however due to the morphological similarity between these species, in my study they were placed in a mixed pool making actual numbers of each unattainable. Unlike *Ae. canadensis*, the adults of these two species began to emerge as early as June 10 but were not in abundant numbers until July (Figure

3.1). Activity appears to be sporadic for *Ae. punctor* and *Ae*. *abserratus* which could be attributed to the staggering behaviour of adult eclosions as part of both lifecycles or due to unfavorable habitat conditions, which could initiate dormancy (Reiter, 1988). Periods of dormancy do not prevent CSV from replicating in the vector host, so the viral load continues to increase during this time. Also, *Ae*. *abserratus* deposits eggs throughout the season in shallow pools that may periodically dry out, which would delay the eggs from hatching and thus delay the eclosure of the adults. As such, in order to anticipate the temporal window of infection with more precision it would be useful to look at the effect of environmental factors, in particular precipitation and temperature (Reiter, 1988). Based on the literature and the data from this study, I would conclude that the overwintering portion of these lifecycles contributes to the persistence of SSHV, likely due to transovarial transmission that is typical of the *Aedes* vector species (Rosen, 1987).

The testing of serum samples from snowshoe hares and sentinel rabbits also contributed to the data on the temporal distribution of SSHV and JCV by establishing the occurrence of seroconversion. The 2010 paired sera and positive PRNT results to IgM (initial response) and IgG (secondary response) antibodies from hare number 2 confirms seroconversion for both SSHV and JCV after August 10 and before September 7; this falls within the same temporal window of SSHV circulation established in the mosquito vectors in this study the following year. Hare number 3 underwent seroconversion for SSHV after May 21 and before June 10. Together these findings suggest there may be a bimodal initiation of infection in the hare population on the Avalon Peninsula for SSHV. Infection can be detected by the appearance of IgM antibodies in the serum with the onset of illness and can persist for months (Calisher, 1994); however, infection by both of these viruses can be asymptomatic and in relation to hares and rabbits the

opportunity may not present itself to observe illness. The IgG antibody response will appear with acute onset of illness, following the presence of IgM, and into the recovery phase (Calisher, 1994). Serology for hare number 3 remained positive for subsequent samples until the negative result on October 7, 2010; this would suggest that the level of IgG antibodies had dropped to an undetectable level. The PRNT will test for overall antibodies, but it has a higher affinity for IgG than IgM (Dimitrova et al., 2010). If IgM was still present in the serum, the result could appear as a false negative, however, IgM can persist in the body for several months, so this still suggests that the infection was clearing (Dimitrova et al., 2010; Makowski et al., 2009). Positive results of anti-JCV and anti-SSHV antibodies in hares 1 and 5, and anti-SSHV antibodies in hare number 4, also confirm the presence of both viruses in the hare population. However, each of the three hares tested seropositive with the first serum sample meaning seroconversion had already occurred, likely during the previous season (2009). The collection of blood from the snowshoe hares was initially planned for biweekly sampling in order to minimize their stress due to handling. Samples were in fact obtained sporadically because of the limitations caused by difficulty in capturing the animals and due to predation and escape of hares from enclosure number 1, which also decreased the sample size. In the 1980-1983 study, 54.5% of the hares that were tested in Terra Nova National Park (central Newfoundland) were seropositive for SSHV and seronegative for JCV (Mokry et al., 1984).

To refine the sample period and to ensure a seronegative start condition, sentinel New Zealand white rabbits were used during the 2011 field season. I was able to handle the rabbits from an early age (3 months old), which helped to reduce their stress while collecting blood samples after they were placed in the field. This meant that the rabbits could be sampled at regular weekly intervals creating a smaller window for determining the time of seroconversion.

Serology for all rabbits was negative for anti-SSHV and anti-JCV antibodies prior to being put in the field; this was tested in order to establish the point of seroconversion for any rabbits that contracted the viruses. One of the 15 rabbits resulted in a clear seroconversion to anti-SSHV antibodies during the week of August 1 to August 9, 2011 at SNP. This window of infection in the sentinel rabbit occurred two weeks prior to the timing of SSHV RNA in the four mosquito species in Outer Cove (*Ae. canadensis*, *Ae. pionips*, *Ae. punctor*, and *Ae. abserratus*). The window of infection appears to be longer when assessing the complete cycle between vector and host, as opposed to assessing the presence of the virus in either the vector or the host in isolation. Another possibility is that this result could include differences in micro climatic factors that were not tested in this study. Keeping in mind that the mosquito vector can also be a reservoir of CSV through transovarial transmission (Rosen, 1987), and that hares are also acting as a reservoir (as suggested by the positive results for hares number 1, 4, and 5), it seems most probable that the window of infection is determined by the life cycle of the mosquito, which dictates when a blood meal is sought. Based on the results for 2011, the temporal window of infection of SSHV in Newfoundland was August 1 and potentially would continue until the end of the mosquito season; no mosquitoes were found flying after the week of September 17. Various contributing factors will alter this time frame from year to year, including the environmental factors that cause mosquitoes to lay dormant, resulting in extended timing of the life cycle and an increased viral load to be transmitted once the mosquito resumes flight (Reiter, 1988).

Within a few days of a mosquito taking in an infected blood meal, the virus migrates from the stomach to the salivary glands, and replicates sufficiently to infect the next mammalian host of the vector (Calisher, 1994). The histological results for rabbit number 1 in this study did not confirm the SSHV infection that was identified from the serological results. In Montana in

August 1959, the first isolation of SSHV was from the blood taken from an infected snowshoe hare and injected into suckling mice (Burgdorfer et al., 1961). Suspensions of spleen and liver tissue from the mice were then inoculated into new mice, killing all within three days, and from these the virus was isolated from the brain tissue. The virus was shown to manifest in spleen, liver, and brain tissue, (Burgdorfer et al., 1961), and a typical arbovirus infection will result in cellular destruction (Mullen and Durden, 2002). Cellular destruction was not apparent in the tissue cultures in my study and so did not warrant further investigation. Although JCV is present on the Avalon Peninsula of Newfoundland, as indicated by the positive serology of hares 1, 2, and 5, the temporal distribution of CSV in the mosquitoes on the island can only be considered for SSHV in this study.

The CSV circulation in Newfoundland has not been restricted to mosquitoes and hares as Mokry and colleagues also documented seropositivity for both JCV and SSHV antibodies in Newfoundland horses and humans. Horses were 18.6% positive for anti-JCV antibodies and 2.3% positive for anti-SSHV antibodies. The human sera tested from rural regions of the Island were primarily seropositive for anti-JCV antibodies at 6.5%, with an additional 1.1% being seropositive to both viruses, and only 0.9% seropositive for anti-SSHV antibodies. The findings of infected wild and domestic animals have more recently been confirmed by Greg Goff and colleagues (2012) who established seroprevalence to SSHV and JCV in bovines, mink, domestic horses, and snowshoe hares, using serum samples taken in 2008 and also from an archive of sheep sera from 1997. Specifically, anti-SSHV antibodies were detected in all five groups of animals, with the highest proportion found in the snowshoe hares and the lowest in the bovines; anti-JCV antibodies were detected in four of the five animals groups, with the highest proportion found in horses, followed by bovines and sheep, and none in the snowshoe hares. The

domesticated mink showed very little seropositivity to either CSV. The findings concluded that both of these CSV are in fact circulating in wild and domestic animals across Newfoundland, emphasizing the potential for related economic impacts to the dairy and sheep farming industries, not to mention human health impacts, should an outbreak of infection occur.

While incidental hosts do not contribute to the CSV reservoir, as they are considered dead-end hosts (Bates, 1965), they are still of concern, particularly in relation to human health. It was, in fact, the clinical cases of infection in Quebec and Ontario in 1978, and Nova Scotia in 1981, which sparked an interest for CSV research in Newfoundland at that time. Two of the three cases in Quebec were determined to be SSHV, with the onset of symptoms occurring June 24, including severe headache, fever and vomiting (Fauvel et al., 1980). One patient also experienced agitation and confusion before the infection cleared almost three weeks later. The third case was a confirmed JCV infection originating on August 7 with the same set of initial symptoms, all of which subsided by day 9. In a location closer to the site of the work presented in this paper, a 5 year old Nova Scotia boy experienced severe headaches, fever, and vomiting, which progressed to seizures and unresponsiveness; the symptoms lasted for 10 days and were diagnosed as SSHV infection (Embil et al., 1982). Looking more closely at the human health concerns, the persistence of SSHV and JCV infections were identified in nearly 10% of the sera samples submitted to NML during the summer of 2008 (Makowski et al., 2009). As well, a random set of serum samples collected from residents of Manitoba between 2004-2007, that were seronegative for WNV, were then retested for CSV. More than 10% were seropositive for IgM antibodies to both SSHV and JCV. At least some of the patients that tested positive experienced fever and seizures and in some cases the patient was diagnosed with meningitis. As mentioned above, humans are a dead-end host and as such do not contribute to CSV

transmission, but because of this it is essential that we do not overlook the ecological parameters that do contribute to CSV circulation and do consider what is happening to the viruses themselves.

Another factor to consider when thinking of vector-borne diseases as moving targets is the speed of viral evolution. In particular for Bunyaviruses, mutations occur regularly, either by genetic drift or genetic shift (Reese, et al., 2008). The absence of a proofreading enzyme during the replication process of single-stranded RNA viruses leaves them vulnerable to point mutations that go undetected and uncorrected, leading to genetic drift (Reese et al., 2008). The well-studied Bunyavirus, La Crosse virus (LACV), is genetically similar to SSHV and causes the most cases of virus-related encephalitis in children in the United States (Rust et al., 1999). When closely related Bunyaviruses infect the same vector species, segment reassortment can occur between the multiple viruses; alternatively, infected offspring (via transovarial transmission) can become re-infected with another closely related virus and undergo the same process of segment reassortment (Beaty et al., 1985). The resultant genetic shift can manifest changes by increasing potential host-range, or can also affect the level of virulence of the viruses. There are geographic regions of overlap between JCV, SSHV, and LACV, such as New York State, and potentially Minnesota and other northern states that border Canada, in which this reassortment would most likely take place (Rust et al., 1999). The primary vector species of LACV is *Aedes triseriatus*, and although LACV has been limited to northern states of the U.S.A., the mosquito vector has been established in the southern regions of many provinces of Canada since at least the 1960's (Steward and McWade, 1961). To date, the primary vector for LACV has not been collected in Newfoundland and thus my mosquito samples were not tested for LACV, nor has evidence of LACV infection been detected in sera samples. Therefore such a genetic shift in SSHV is

unlikely in Newfoundland at this point. Because LACV is highly cross-reactive with SSHV in PRNT assays, members of the zoonosis lab at NML tested for SSHV only in the snowshoe hare and rabbit sera samples for 2010 and 2011. Also, the primary vertebrate hosts of LACV are grey squirrels and eastern chipmunks (Calisher, 1994), and not snowshoe hares and rabbits. Genetic drift occurs regularly as a consequence of point mutations in the virus RNA genome during replication, but selection usually occurs for the most advantageous of these mutations (Forrester et al., 2012). When a deleterious, irreversible genomic mutation occurs that affects the fitness of the population, it can be attributed to a small population size that is associated with isolation. The isolation of Newfoundland from the mainland could provide the opportunity for such genetic drift to occur in the SSHV, which could lead to changes in fitness of the virus, as well as diversity of the virus (Holland et al., 1982). This potential contributes to the importance of characterizing the SSHV that was isolated in the two mosquito samples that were collected in 2011.

Members of the zoonosis lab at NML were able to characterize a 579 nucleotide sequence (KB 111) of SSHV RNA from the *Ae*. *canadensis* pool and a 200 nucleotide sequence from the second pool (KB 112) containing the mix of *Ae*. *pionips*, *Ae*. *punctor*, and *Ae*. *abserratus*. The smaller recovered portion for KB 112 may have been due to lower amounts or diminished integrity of the SSHV RNA in that sample. The region of overlap was identical, so KB 111 was used in the comparison analysis with SSHV RNA isolates from other geographic locations. The SSHV RNA sequence from sample KB 111 is from the S segment of the genome (from nucleotide 323 to 901) and was compared to four distinct SSHV clades: the prototype Burg 59, Ont 61, Yukon 80, and Sask 93. The S segment has been found to be the most highly conserved region of the genome, making it a suitable target for amplification and appropriate for genomic

analysis (Elliot, 1990). The Newfoundland sequence KB 111 is 98% conserved with that of the Burg 59 sequence, which is the prototype strain from a snowshoe hare blood sample from Montana in 1959 (Burgdorfer, et al., 1961). The KB111 sequence is also highly conserved with Ont 61, at 97%, and with the Yukon 80 strain, at 95%. Considering that KB 111 has very little variation compared to those sequences of the mainland, it appears that only limited genetic drift has taken place in this region of the genome since the virus became distributed across North America. The greatest divergence between the KB111 sequence and the reference sequences was with that of Sask 93, from Saskatchewan, at 83.7% identity. Saskatchewan is geographically closer to the potential areas of overlap between different Bunyaviruses mentioned earlier, in particular Minnesota, so perhaps this greater variation can be attributed to genetic reassortment. This comparison shows a variation of SSHV strains across Canada, from 1959 to 2011. The introduction of the virus to Newfoundland presumably predates 1983 when it was originally found here, and it likely remains conserved since this introduction. However, sequence data from more of the virus genome and from more viruses throughout time would be required to properly understand historical movements of the virus. The implications of my study show that there is no reason to doubt that the SSHV and JCV detected in the 1980-1983 study (Mokry et al., 1984) have persisted on the Island over the subsequent decades.

More research is needed on factors that regulate the life-cycles of the four candidate vectors of SSHV identified in this study, as well as the mosquito species that have been identified in other studies as being vectors of JCV. These factors likely include climatic changes, annual precipitation and temperatures which could provide forecasts of what the next mosquito season is likely to hold. Furthermore, it would be of interest to examine the geographical analysis of Goff et al.,)2012) in developing an expanded distribution of sentinel rabbits in various

hotspots that they have identified with a focus on establishing a database of temporal infection activity coupled with vector analysis in the same areas.

The dynamic components involved in transmission of mosquito-borne viruses means that ongoing surveillance is necessary. This includes not only what has already been identified as being present on the Island, but also must include species which have potential as CSV vectors in the future. One such moving target of potential pathogenesis, *Cx. pipiens*, was identified on the west coast of Newfoundland in 2006 by Sarah Hustins, and again on the east coast by me in 2012. *Culex pipiens* is a primary vector of WNV in North America north of the 39th parallel. However, there is no evidence to date that WNV is present on the Island (Hustins, 2006). While trying to establish the distribution of vectors and their pathogens continues to be a challenge it remains an integral part of understanding the epidemiology of infectious diseases.

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6 APPENDICES

6.1 Appendix A: Correlation analyses and frequency analyses of variables affecting likelihood of trap catching mosquitoes, based on 2010 field results.

The placement of 10 dry-ice baited CDC miniature light traps for the use of collecting mosquitoes in 2010 at Salmonier Nature Park, were based on the trap manufacturing protocol in terms of height and proximity to buildings, and were also based on proximity to the two snowshoe hare enclosures in the Park. In consideration of possible relationships between trap location variables (habitat) and environmental variables, on the likelihood of successful trapping of mosquitoes, I performed correlation analyses. The results of these analyses were used to improve the placement of the mosquito traps at SNP during the 2011 field season.

Hypothesis 1: Habitat variables affect the likelihood of a trap catching mosquitoes

Hypothesis 2: Weather variables affect the likelihood of a trap catching mosquitoes

Habitat variables:

Shrubs abundant	Yes/No
Fern abundant	Yes/No
Height of Trap	Yes/No
Grass Abundant	Yes/No
Moss Abundant	Yes/No
Water within 3 meters	Yes/No
Tree circumference	Yes/No
Height of Trap 1.52 meters	Yes/No
Height of Trap 1.37 – 1.4 meters	Yes/No
Height of Trap 1.06 meters	Yes/No
Relative Elevation	Yes/No
Dense Canopy	Yes/No

Weather Variables:

Temperature
Relative Humidity
Wind Direction
Wind Speed
Rainfall

Results of Pearson Correlation Analyses:

Table 1: Testing of **Hypothesis 1** using a correlation analysis in SPSS to examine the relationship between habitat variables and the success of mosquitoes being caught in the traps.

Habitat variable	**Correlation Coefficient**	**Significance**
Shrubs abundant	-0.80	0.346
Fern abundant	0.131	0.123
Height of Trap	**-0.291**	**** 0.000**
Grass Abundant	-0.80	0.346
Moss Abundant	**0.226**	**** 0.007**
Water within 3 meters	-0.058	0.494
Tree circumference	0.004	0.958
Height of Trap 1.52 meters	**-0.250**	**** 0.003**
Height of Trap 1.37–1.4 m	0.117	0.170
Height of Trap 1.06 meters	**0.214**	** 0.011**
Relative Elevation	-0.071	0.402
Dense Canopy	**0.216**	**** 0.010**

Habitat variables that have a significant correlation to the catch of mosquitoes in the traps.
All correlations, however, are weak.
* Sig at 0.05 level (2-tailed)
** Sig at 0.01 level (2-tailed)

Table 2: Testing of **Hypothesis 1** using a correlation analysis in SPSS to examine the relationship between habitat variables and the rate of catch per trap.

Habitat variable	Correlation Coefficient	Significance
Shrubs abundant	**-0.193**	*** 0.022**
Fern abundant	**0.461**	**** 0.000**
Height of Trap	**-0.316**	**** 0.000**
Grass Abundant	-0.066	0.438
Moss Abundant	**0.473**	**** 0.000**
Water within 3 meters	-0.132	0.119
Tree circumference	0.042	0.622
Height of Trap 1.52 meters	**-0.440**	**** 0.000**
Height of Trap 1.37 – 1.4 meters	**0.458**	**** 0.000**
Height of Trap 1.06 meters	-0.044	0.605
Relative Elevation	0.162	0.056
Dense Canopy	**0.309**	**** 0.000**

Habitat variables that have a significant correlation to the rate of catch per trap.
Moss abundance, fern abundance, and height of trap 1.37-1.4 m, have strong correlation; height of trap 1.52 m also has a strong, but negative correlation. Dense canopy and height have medium correlations; and the abundance of shrubs has a weak correlation

***** Sig at 0.05 level (2-tailed)

****** Sig at 0.01 level (2-tailed)

Table 3: Testing of Hypothesis 2 using a correlation analysis in SPSS to examine the relationship between weather variables and whether or not mosquitoes are caught in the trap.

Weather Variables	Correlation Coefficient	Significance
Temperature	**0.210**	*** 0.013**
Relative Humidity	-0.138	0.104
Wind Direction	0.042	0.623
Wind Speed	-0.011	0.900
Rainfall	-0.103	0.225

Weather variables that have a significant correlation to mosquitoes caught or not caught.
Temperature has a positive correlation, but it is weak.
*Significance at 0.05 level (2-tailed)

Table 4: Testing Hypothesis 2 using a correlation analysis in SPSS to examine the relationship between weather variables and the rate of mosquitoes caught per day.

Weather Variables	Correlation Coefficient	Significance
Temperature	**0.290**	**** 0.001**
Relative Humidity	**-0.231**	**** 0.008**
Wind Speed	-0.041	0.645
Rainfall	-0.166	0.060

Weather variables that have a significant correlation to the rate of mosquitoes caught per day.

Temperature, in this analysis, has a moderately positive correlation to the ratio of mosquitoes caught per day; whereas relative humidity is slightly less than moderate, but negatively correlated.

****Significance at 0.01 level (2-tailed)

Results of Frequency Analyses:

Based on the results of the correlation analyses above, frequency analyses were carried out on significant variables, with a focus on the positive correlations.

- **Dense Canopy:** A dense canopy increases the rate of mosquitoes caught per trap, at a frequency of 70%.
- **Moss:** The presence of moss increases the rate of mosquitoes caught per trap, at a frequency of 80%.
- **Fern:** The presence of fern increases the rate of mosquitoes caught per trap, at a frequency of 60%.
- **Height:** The height of a trap at 1.37-1.4 meters has a frequency of 50/50 for catching or not catching mosquitoes; as opposed to the height of 1.52 meters which had a frequency of 40% caught vs. 60% not caught; and the height of 1.06 meters had a frequency of 10% caught vs. 90% not caught.
- **Water within 3 meters:** The presence of water within 3 meters of the trap has a 50/50 chance of affecting the rate of mosquitoes caught per trap.

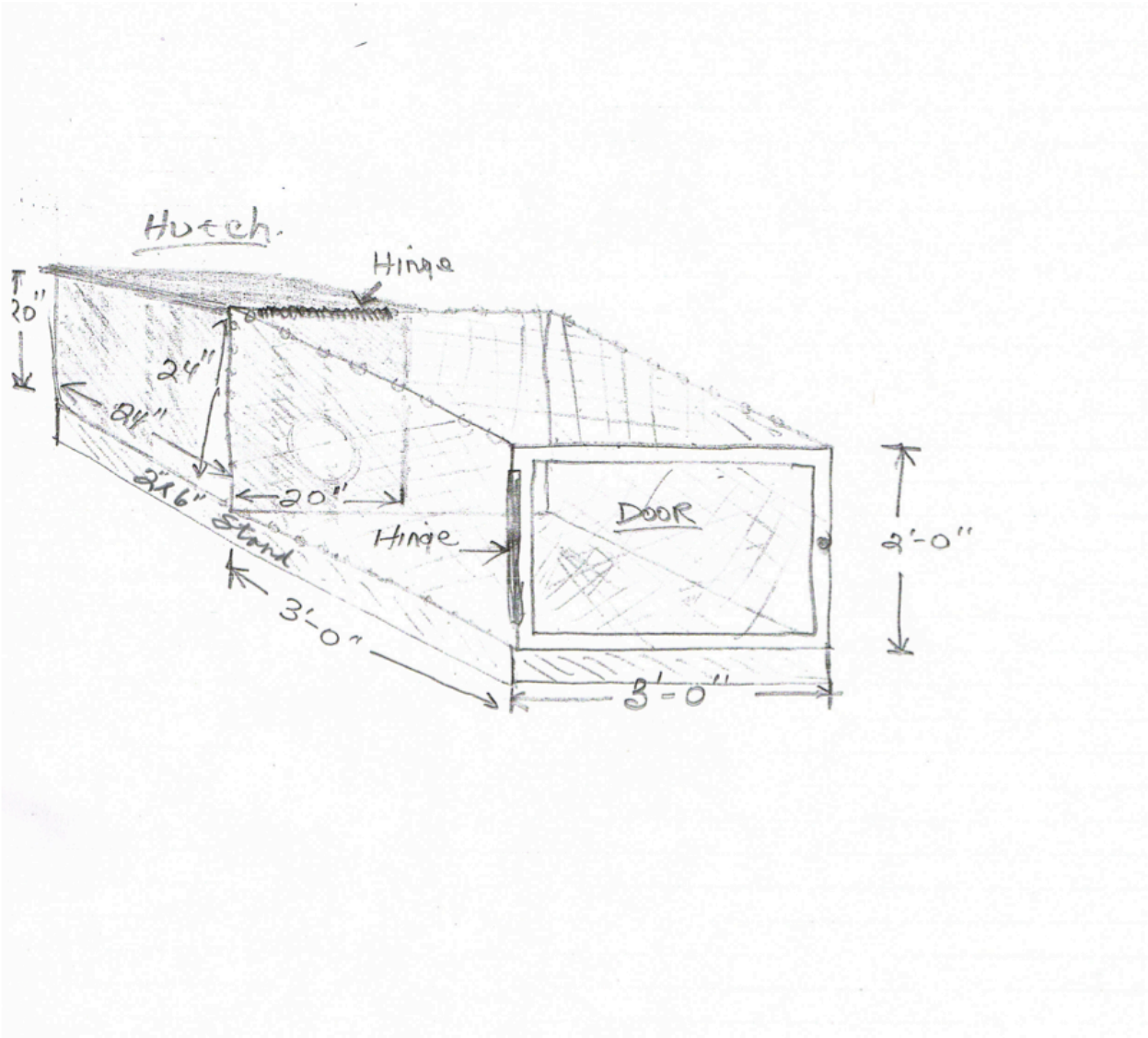
Conclusions:

The habitat and weather variables that affect the likelihood of a trap catching mosquitoes:

- Dense Canopy
- Abundant Moss/Fern
- Temperature

The placement of traps at SNP during the 2011 field season, was based on the above correlation and frequency analyses of 2010 field season variables and the likelihood of the traps catching mosquitoes. Traps were set under a dense canopy of trees in an area of high moisture (as evidenced by the presence of moss and fern). The effect of temperature on the rate of mosquitoes caught per day was highly significant, with the rate increasing when temperatures were above 15^{o}C. Regarding height, the results show that placing the trap at 1.37– 1.40 m above ground had a 50/50 frequency between catching and not catching mosquitoes. This is an improvement over the frequency of successful trapping when the height is 1.52 meters (40%) or 1.06 meters (10%).

6.2 Appendix B: Rabbit hutch sketch designed by Salmonier Nature Park Carpenter, 2011.



6.3 Appendix C: Snowshoe hare virus RNA sequence:

From: Bishop, D. H. L., K. G. Gould, H. Akashi, and C. M. Clerx-van Haaster. (1982). The complete sequence and coding content of snowshoe hare Bunyavirus small (S) viral RNA species. Nucleic Acids Research, 10(12): 3703-3713.

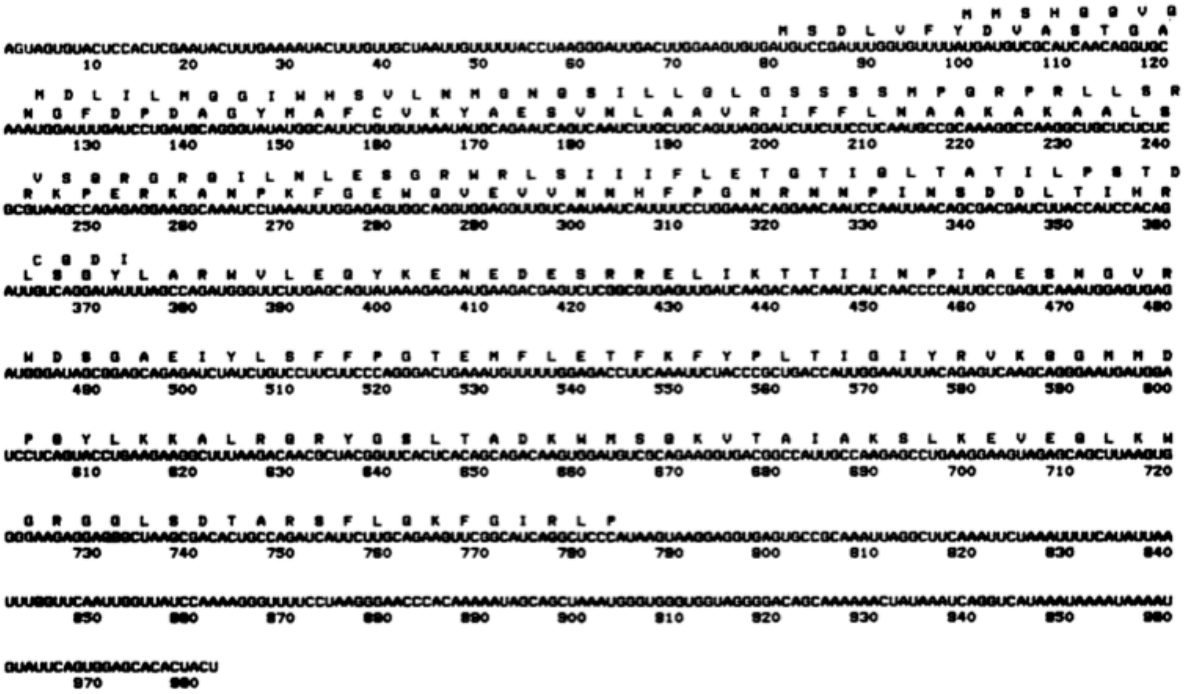


Fig. 2. The nucleotide and amino acid sequences deduced for SSH S viral RNA species. The RNA sequence, written as the complementary (plus sense) strand, was deduced from analyses of a DNA copy cloned in pBr322 (S clone 17, see text), in addition to direct RNA and dideoxy sequence data (9,10). The nucleotides are numbered below their respective residues. Amino acids of polypeptides (coded by contiguous open reading frames and initiated by methionine codons) are centered over the corresponding nucleotide triplets.

6.4 Appendix D: Nucleic Acid sequence for Newfoundland snowshoe hare virus (SSHV) isolate KB 111; and alignment with four distinct SSHV clades.

RNA Sequence: KB 111

AACAATCCAATTAACAGCGACGATCTTACCATCCACAGATTGTCAGGATATTTAGCCAGATGGGTTCTTGAGCAGT
ATAAAGAAAATGAGGATGAGTCTCGGCGTGAGTTGATCAAGACAACAATCATCAACCCCATTGCCGAGTCGAACG
GAGTGAGATGGGATAGCGGAGCAGAGATCTATCTGTCCTTCTTCCCAGGGACTGAAATGTTTTTGGAAACCTTCAA
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AGACAACGCTATGGTTCACTCACAGCAGACAAGTGGATGTCGCAGAAGGTGACAGCCATTGCCAAGAGCCTGAAA
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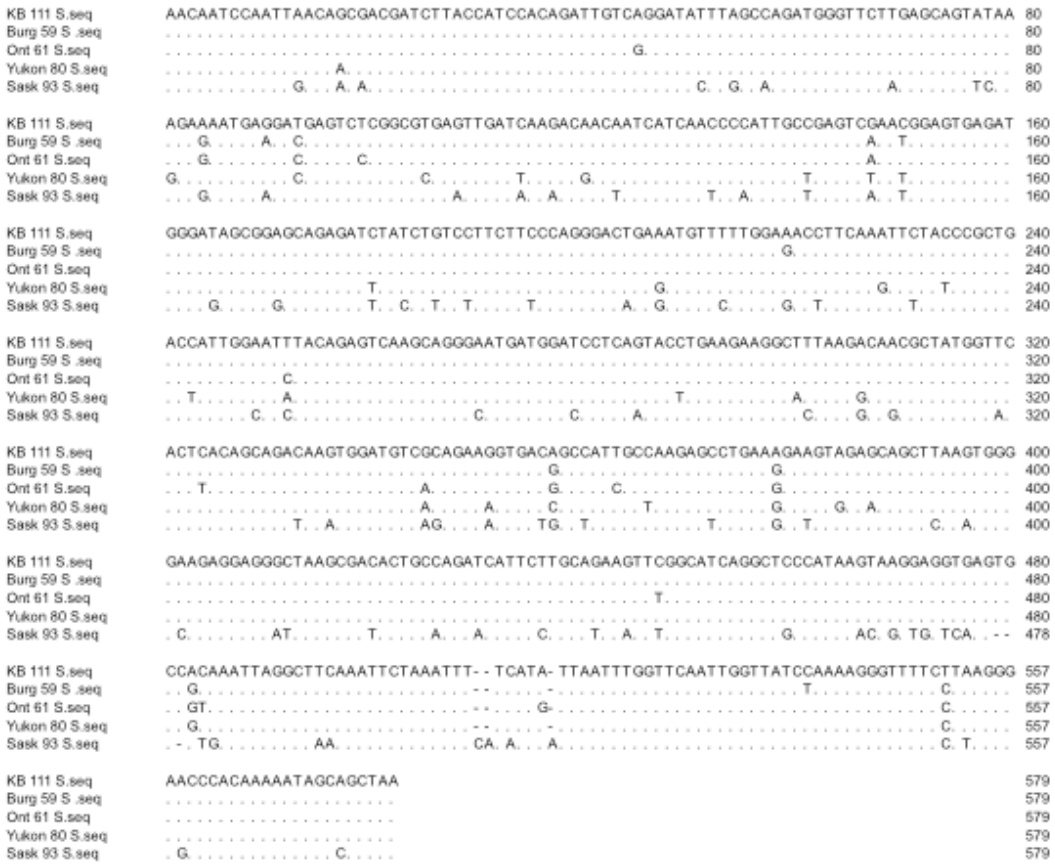
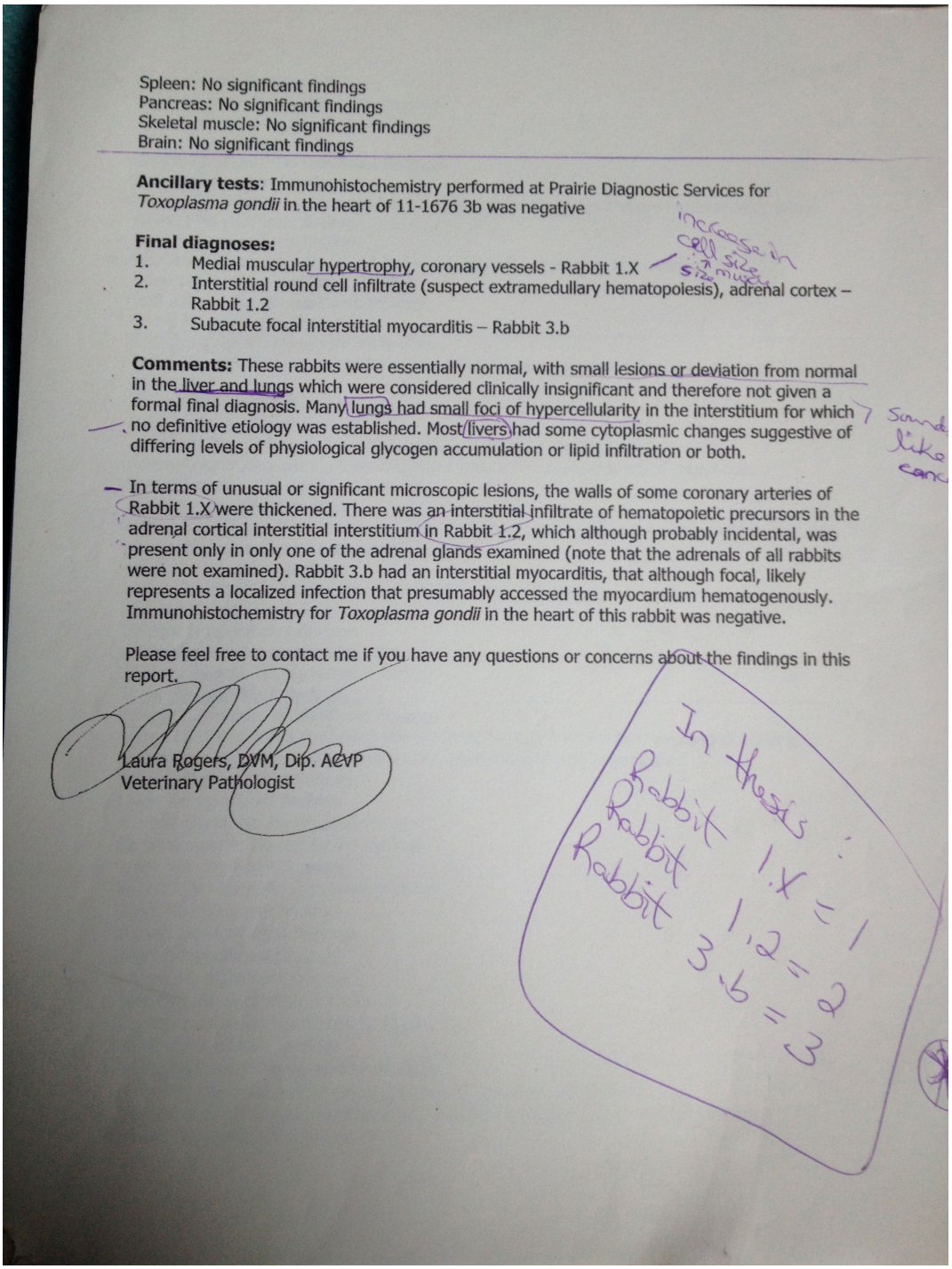
TGGTTATCCAAAAGGGTTTTCTTAAGGGAACCCACAAAAATAGCAGCTAA



6.5 Appendix E: **Histopathology Report by Dr. Laura Rogers**



Spleen: No significant findings
Pancreas: No significant findings
Skeletal muscle: No significant findings
Brain: No significant findings

Ancillary tests: Immunohistochemistry performed at Prairie Diagnostic Services for *Toxoplasma gondii* in the heart of 11-1676 3b was negative

Final diagnoses:

1. Medial muscular hypertrophy, coronary vessels - Rabbit 1.X
2. Interstitial round cell infiltrate (suspect extramedullary hematopoiesis), adrenal cortex – Rabbit 1.2
3. Subacute focal interstitial myocarditis – Rabbit 3.b

Comments: These rabbits were essentially normal, with small lesions or deviation from normal in the liver and lungs which were considered clinically insignificant and therefore not given a formal final diagnosis. Many lungs had small foci of hypercellularity in the interstitium for which no definitive etiology was established. Most livers had some cytoplasmic changes suggestive of differing levels of physiological glycogen accumulation or lipid infiltration or both.

In terms of unusual or significant microscopic lesions, the walls of some coronary arteries of Rabbit 1.X were thickened. There was an interstitial infiltrate of hematopoietic precursors in the adrenal cortical interstitial interstitium in Rabbit 1.2, which although probably incidental, was present only in only one of the adrenal glands examined (note that the adrenals of all rabbits were not examined). Rabbit 3.b had an interstitial myocarditis, that although focal, likely represents a localized infection that presumably accessed the myocardium hematogenously. Immunohistochemistry for *Toxoplasma gondii* in the heart of this rabbit was negative.

Please feel free to contact me if you have any questions or concerns about the findings in this report.

Laura Rogers, DVM, Dip. ACVP
Veterinary Pathologist

Pancreas: No significant findings
Adrenal gland: No significant findings
Skeletal muscle: No significant findings
Brain: No significant findings

Rabbit 2.5: Heart: No significant findings
Lung: No significant findings
Liver: Centrilobular hepatocytes have a moth eaten cytoplasm and are moderately swollen.
Kidney: No significant findings
Spleen: No significant findings
Pancreas: There are a few small foci of fat necrosis adjacent to the pancreas
Adrenal gland: No significant findings
Skeletal muscle: No significant findings
Brain: No significant findings

Rabbit 3.a: Heart: No significant findings
Lung: The perivascular adventitia of some small veins contains increased numbers of heterophils.
Kidney: No significant findings
Spleen: No significant findings
Pancreas: The peripancreatic adipose tissue contains a few foci of mineralization
Adrenal gland: The cortical interstitium contains a few loose clusters of round cells.
Skeletal muscle: No significant findings
Brain: No significant findings

Rabbit 3.b: Heart: The interstitium is focally expanded by moderate numbers of lymphocytes, macrophages and fewer heterophils.
Lung: No significant findings
Liver: Centrilobular hepatocytes have a moth eaten cytoplasm.
Kidney: No significant findings
Spleen: No significant findings
Adrenal gland: No significant findings
Skeletal muscle: No significant findings
Brain: No significant findings

Rabbit 2.2: Heart: No significant findings
Lung: The interstitium contains a few small foci of hypercellularity composed of varying proportions of lymphocytes, plasma cells, macrophages and heterophils.
Kidney: No significant findings
Liver: No significant findings
Adrenal gland: No significant findings
Skeletal muscle: No significant findings
Brain: No significant findings

Rabbit 2.3: Heart: No significant findings
Lung: No significant findings
Kidney: No significant findings
Liver: No significant findings
Spleen: No significant findings
Pancreas: No significant findings
Skeletal muscle: No significant findings
Brain: No significant findings

Rabbit 2.4: Heart: No significant findings
Lung: The muscular wall of some pulmonary arteries is mildly thickened.
Liver: Most hepatocytes are moderately swollen and have a moth eaten cytoplasm.
Kidney: No significant findings

Brain: No significant findings

Rabbit 1.3: Heart: No significant findings
Lung: No significant findings
Kidney: No significant findings
Liver: Many hepatocytes, most prominently in centrilobular areas, are mildly swollen and have a moth eaten cytoplasm.
Spleen: No significant findings
Skeletal muscle: No significant findings
Brain: No significant findings

Rabbit 1.5: Heart: No significant findings
Lung: The interstitium contains 2 small to medium sized foci of hypercellularity composed of heterophils and macrophages, that sometimes spill into the surrounding alveoli.
Liver: Most hepatocytes are mildly swollen and have a moth eaten cytoplasm.
Kidney: No significant findings
Spleen: No significant findings
Pancreas: No significant findings
Adrenal gland: No significant findings
Skeletal muscle: No significant findings
Brain: No significant findings

Rabbit 2.1: Heart: No significant findings
Lung: The interstitium contains a small focus of hypercellularity composed of macrophages, lymphocytes and occasional heterophils.
Liver: A few round cells are present in portal connective tissues.
Kidney: No significant findings
Spleen: No significant findings
Adrenal gland: No significant findings
Skeletal muscle: No significant findings
Brain: No significant findings

Rabbit 3.1: Lung: The interstitium contains a few small foci of hypercellularity composed of round cells and a small amount of karyorhectic debris.
Liver: No significant findings
Spleen: No significant findings
Pancreas: No significant findings
Adrenal gland: No significant findings
Skeletal muscle: No significant findings
Brain: No significant findings

Rabbit 3.3: Heart: No significant findings
Lung: No significant findings
Liver: Portal connective tissue contains a few round cells.
Kidney: No significant findings
Spleen: No significant findings
Adrenal gland: No significant findings
Pancreas: No significant findings
Skeletal muscle: No significant findings
Brain: No significant findings

Rabbit 3.4: heart: No significant findings
Lung; No significant findings
Liver; Many hepatocytes, particularly those in centrilobular areas, are mildly swollen with a moth eaten cytoplasm.
Kidney: No significant findings
Spleen: No significant findings

PRAIRIE DIAGNOSTIC SERVICES (966-7316)
Western College of Veterinary Medicine
U of S, 52 Campus Dr, Saskatoon, SK S7N 5B4

D1202996

PLEASE DO NOT PAY -- PAY FROM INVOICE

Submitted: 2-Feb-12 Case:
Collected:

Total Amount: $52.50
[Pending: $0.00] Invoiced: $52.50

Copy to Dr: Sparkes

Copy to:

DEPARTMENT OF NATURAL RESOURCE
PROVINCIAL AGRICULTURE BUILDING
ST.JOHN'S, NF
A1E 3Y5

Phone: (709) 729-6879 Fax: (709) 729-0055

Owner: 11-1676 3b-1
Animal: 11-1676 3b-1
Species: Rabbit (Hare)
Sex: Unknown
Age: Unknown
Clinician:
Pathologist:
Submitted: 1 Type: Paraffin Block
For: Immunology $50.00, Histo Lab , GST $2.50

Immunology FINAL

SPECIMEN(S) SUBMITTED:

Formalin tis.sections x 1

RESULTS & INTERPRETATION: 7-Feb-12

ID	Specimen	Examination	Method	Diag/Titre
	Formalin tis.sections [11-1676-36-1]	Toxoplasma gondi	Avidin biotin complex - Peroxidase	Negative

Toxoplasma organisms were not detected in the heart.

Supervisor: GODSON,DALE