Characterization of the Fecal Virome and Fecal Virus Shedding Patterns of Commercial Mink (*Neovison vison*)

by

Xiao Ting (Wendy) Xie

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ABSTRACT

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Wendy Xie
University of Guelph, 2017

Advisor:
Dr. Patricia V. Turner

This study characterized the mink fecal virome using next-generation sequencing and investigated fecal shedding of mink-specific astrovirus, rotavirus and hepatitis E virus (HEV) over 4-years, using pooled fecal samples from commercial adult females and kits. Sequencing of 30 female and 37 kit pooled fecal samples resulted in 112,144 viral sequences with similarity to existing genomes. Of 109,612 bacteriophage sequences, *Escherichia* and *Enterococcus*–associated phage (16% and 11%, respectively) were most prevalent. Of 1237 vertebrate sequences, viral families *Parvoviridae* and *Circoviridae* were most prevalent，and 27% of viral sequences identified were of avian origin. Astrovirus, rotavirus, and HEV were detected in 14%, 3%, and 9% of samples, respectively. HEV was detected in significantly more kit than female samples ($p<0.0001$), and astrovirus in more summer samples than winter samples ($p=0.001$). This research permits improved understanding of potential causative agents of mink gastroenteritis, as well as virus shedding in healthy commercial mink.

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谢谢爸爸妈妈的爱，谢谢你们一直在支持我，鼓励我。- 你们的乖女儿

DECLARATION OF WORK PERFORMED

All work presented in this thesis was completed by myself, with the following exceptions:

- Collection of fecal samples was completed by Brian Tapscott (OMAFRA)
- Initial receipt of fecal samples was conducted by Dr. Jutta Hammermeuller
- Virus-specific PCRs were done in part by summer students Rachel MacDonald, Brianne Mercer, Sabrina Stevens, and Deidre Stuart
- Culture and susceptibility testing was conducted by Nicol Janecko at the Laboratory for Foodborne Zoonosis in Guelph, ON, a branch of the Public Health Agency of Canada, Ottawa, ON
- Fecal virome sequencing using Illumina NextSeq was conducted by the Donnelly Centre, University of Toronto
- Sequence trimming and assembly was performed using iVirus (CyVerse, AZ USA) and SeqManNGen 12 (DNAStar, Lasergene, WI USA)
- Sequence identification was performed using Geneious 10.1.3 software provided by Dr. Andrew Kropinski
- Identification of antimicrobial resistance genes was performed using the CARD database provided by Dr. J Scott Weese

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ABBREVIATIONS

AAV	Adeno-associated virus
AD	Aleutian disease
ADV	Aleutian disease virus
AdV	Adenovirus
AMR	Antimicrobial resistance
ARG	Antimicrobial-resistant genes
AstV	Astrovirus
CARD	Comprehensive Antibiotic Resistance Database
CAV	Chicken anemia virus
CDV	Canine distemper virus
CoV	Coronavirus
dsRNA	Double stranded RNA
E. coli	*Escherichia coli*
ECG	Epizootic catarrhal gastroenteritis
FeAstV	Feline astrovirus
FHV	Feline herpesvirus
FMDV	Foot-and-mouth disease virus
HAstV	Human astrovirus
HEV	Hepatitis E virus
HPS	Hantavirus pulmonary syndrome
MCoVs	Mink *Alphacoronoviruses*
MEV	Mink enteritis virus
MiAstV	Mink astrovirus
MpPV1	*Mustela putorius furo* papillomavirus
NCBI	National Centre for Biotechnology Information
NGS	Next-generation sequencing
NS1	Nonstructural 1
OAHN	Ontario Animal Health Network
OAstV-1	Ovine mink astrovirus
ORF	Open reading frames
PCRs	Polymerase-chain reactions
RdRp	RNA-dependent RNA polymerase

RV	Rotavirus
SARS	Severe acute respiratory syndrome
SMS	Shaking mink syndrome
SNV	Sin Nombre virus
ssRNA	Single stranded RNA
VA/HMO	Virginia/human-mink-ovine-like astrovirus
VP1	Viral protein 1

Chapter 1: Viral metagenomics in mink: important eukaryotic viruses and bacteriophage and their roles in mink health

1.1 Introduction

This research aims to investigate the composition of the fecal viral biome of commercial mink (*Neovison vison*) in Ontario. Many common diseases of commercial mink result from viral infections and co-infections that are poorly characterized with few validated diagnostic tests. Currently, we have little to no data for the on-farm prevalence for most of the viruses identified to cause disease. In a study on the mortality of farmed mink in western USA, 91.7% of mink deaths were determined to be the result of a single disease (n=339) (Wilson et al., 2015). The top five diseases were found to be bacterial pneumonia (18.8%) which were mainly caused by *Pseudomonas aeruginosa*, Aleutian mink disease (17.7%), mink viral enteritis (16.2%), hepatic lipidosis (8.1%), and nutritional myopathy (7%) (Wilson et al., 2015). On commercial mink farms, outbreaks of infectious diseases can result in high numbers of kit mortality and heavy financial losses for producers. Biosecurity on mink farms is poor, and domestic mink may be exposed to a variety of wild and domestic animal species as well as humans entering the farm (Compo et al., 2017). From a zoonotic perspective, commercial animals may serve as reservoirs of viruses that can be transmitted to farmers and their workers. Metagenomic characterization and quantification of viral infections in commercial animal populations will allow for better understanding of the prevalence of disease-associated viruses and also allow for identification of novel or highly divergent viruses that may be playing a role in health and disease of commercial animals. Overall, the results of this metagenomic project will allow us to assess the diversity of viral infections in Ontario commercial mink, compare the viral infections between adult female mink and mink kits, and assess the differences in viral biome composition between farms and regions. These comparisons can later be correlated to the general health status of the animals and reported cases of outbreaks, vaccinations, farm biosecurity, and diet to help implement practices that will decrease the likelihood of viral outbreaks and economic loss.

1.2 Commercial Mink Farming

In 2015, over 3 million mink pelts were produced in Canada, valued at $98 million (Statistics Canada, 2016). Minks are individually housed in open sheds in wire mesh cages

with an attached nest box. The sheds allow for maximum light and ventilation, both of which can be manipulated by the producer depending on the season. Each cage has a water bowl or water nipple, and wet feed in the form of a patty is usually distributed directly onto the wire top of the cage. Feed is composed mainly of livestock, poultry or fish by-products, eggs, cheese, and approximately 10% grain by-products. Supplemental minerals and vitamins may also be added. Producers can grind and mix their own feed on-farm, which is more labour intensive, but a slightly less expensive process, or buy pre-processed ready-to-use feed at a higher net cost. Large cold storage units are needed if a farmer chooses to process their own feed (NFACC, 2013). Usually, producers in one region will obtain mink feed from one source.

Mink farmers usually breed one mating male per five female breeders. Breeding occurs in February to March, and females may be food restricted during the winter to achieve optimal body condition for breeding and parturition. Boudreau et al. (2014) showed that females with lower body weights are less susceptible to metabolic disorders and produce larger live litters than heavier females. After producing litters, diet restricted females were also better able to maintain body weight during lactation and regain weight and condition after lactation (Boudreau et al., 2014).

Mink are seasonal breeders and are sensitive to the level of light exposure, and artificial lights in the shed may interfere with normal reproductive behaviours. Exposing the females to more hours of light accelerates the gestation period and enhances the reproductive process (NFACC, 2013; Hammond, 1951). Kits are born in April and into early May and are weaned between 6-8 weeks, after which they are pair-housed until pelt-out. Other than the volume of feed offered, there is no difference between the adult and kit diets. The pelt growth period can be expedited by reducing the number of hours the animals are exposed to light. By November or December, the pelts are ready to be collected. Animals to be pelted are euthanized by carbon monoxide inhalation (NFACC, 2013).

1.3 Viral metagenomics in domestic and wild mammal species

Previous viral metagenomic studies have shown that domestic and wild mammal species are shedding many well characterized viruses, but also a wide variety of uncharacterized or highly divergent viruses (Bodewes et al., 2014; Conceição-Neto et al.,

2015; Mihalov-Kovács and Krisztia, 2014; Phan et al., 2011; Smits et al., 2013; Zhang et al., 2014). The use of next-generation sequencing (NGS) and the National Centre for Biotechnology Information (NCBI) viral database have improved the efficiency and ease of total virome characterization and virus identification. These studies have highlighted the importance of using metagenomic methods to improve our understanding of transient viruses in animals that may have direct or indirect contact with the human population regularly, as many of the identified viruses are potentially zoonotic. While fecal virome work is relatively new, studies have been reported for some domestic and wild carnivores, research ferrets, and commercial swine and rabbits.

1.3.1 Felids

Cats often live in close proximity to humans worldwide and many cats can be exposed to a wide variety of viruses in their environment, and in the context of this work, function as a representative carnivorous species (Zhang et al., 2014). Metagenomic studies using NGS methods on the fecal virome of cats allow us to assess zoonotic risk and the role that these viruses play in feline health (Ng et al., 2014; Zhang et al., 2014). Rabies virus is considered a feline zoonotic viral agent (Ng et al., 2014). Additionally, viral metagenomic studies in felids serve as one of the few examples of carnivore virome characterization, and the results of these studies may be instructive for other carnivore species.

In two viral metagenomic studies, known picornavirus, astrovirus, bocavirus, picobirnavirus, rotavirus and coronavirus sequences were found to be most prevalent (Ng et al., 2014; Zhang et al., 2014). Ng et al. (2014) identified a new feline astrovirus, FeAstV Viseu, that was found to induce enteritis in experimental infections of specific-pathogen-free cats (Harbour et al., 1987). FeAstV Viseu had 92.6% identity to the feline astrovirus FeAstV2 from Hong Kong, 70.8% to FeAstV Bristol as well as 58.7% identity to human astrovirus 1. The authors suggested that previous human-cat astrovirus transmission may have occurred because the Viseu, Bristol and Hong Kong feline astroviruses were closely related to human astroviruses (HAstVs) serotypes 1-8 (Ng et al., 2014). These HAstVs are associated with viral gastroenteritis, especially in children, immunocompromised individuals, and the elderly, and transmission of feline astroviruses to humans may have caused an unknown number of enteritis cases (Ng et al., 2014; Vu et al., 2017). Cats have never been considered as a major reservoir for astroviruses that may be transmitted to and cause disease in humans, and these results demonstrate the need to further characterize astroviruses isolated

from human cases of viral enteritis.

A novel bocavirus that had <80% amino acid homology to other known feline bocaviruses (Accessions JQ692585-7) was identified, tentatively named feline bocavirus 2 strain POR1 (FBoV2-POR1, accession KF792837) (Ng et al., 2014). This new bocavirus was phylogenetically clustered with three other bocaviruses reported from Hong Kong with 58% to 70% identities in their NS1 (nonstructural protein 1, plays a major role in parvovirus toxicity in animals) and viral protein 1 (VP1) (Hristov et al., 2010; Ng et al., 2014). Feline bocaviruses are most closely related to canine minute virus and canine bocavirus (Ng et al., 2014). Although their pathogenicity is not well understood, picobirnaviruses have been detected in humans, other mammals, reptiles, and birds (Ng et al., 2014). Ng et al. (2014) reported the first picobirnavirus infection in cats and the family Felidae, and the identified sequence (1236 bp of the RNA-dependent RNA polymerase gene [RdRp]) had 65% identity to a GII human picobirnavirus. Interestingly, a study in the Netherlands found picobirnaviruses in approximately 20% (n=84) of human clinical diarrheal samples (van Leeuwen et al., 2010). Although authors indicated that these viruses may be associated with viral enteritis, only diarrhea samples were analyzed in this study and the prevalence of picobirnaviruses in healthy fecal samples was not explored in this study (van Leeuwen et al., 2010). A novel feline picornavirus was identified and named feline sakobuvirus A (SakoV-A strain FFUP1, accession KF387721) (Ng et al., 2014). It was found to be most closely related to the *Kobuvirus* and *Salivirus* genera (41% and 40%, respectively), therefore Ng et al. (2014) proposed the name *Sakobuvirus* for a novel picornavirus genus. The P1, P2, and P3 regions (non-structural polyprotein associated with genome replication) of this feline sakobuvirus had 37%, 38%, 45% identities, respectively, to the human Aichi virus 1 (*Kobuvirus*) (Cameron et al., 2010; Ng et al., 2014). Finally, a novel rotavirus, feline rotavirus Viseu (Accession KF792839), was found to have 53% amino acid identity to group H rotaviruses and <47% identity to group B rotaviruses, which have been associated with diarrhea in swine (group H) and humans (group B) (Hull et al., 2016; Molinari et al., 2014; Ng et al., 2014; Sanekata et al., 2003). This feline rotavirus Viseu could be a new species of rotavirus, but more analysis of the viral sequences is required to determine how this virus is related to other rotaviruses. Whether feline rotavirus Viseu is associated with diarrhea in cats was not investigated in this study.

A viral metagenomic study conducted by Zhang et al. (2014) had similar results. Sequenced samples collected from Californian shelter cats were found to have viruses with high sequence similarities to the viral families *Astroviridae*, *Coronaviridae*, *Parvoviridae*, *Herpesviridae*, *Caliciviridae*, and *Picobirnaviridae*, but also contained viruses with low levels of similarities to viral families *Parvoviridae*, *Circoviridae* and *Anelloviridae*. The most prevalent viruses were mamastroviruses from the family *Astroviridae* (63.7% of all reads), which have been associated with diarrhea in many species, including humans, swine, dogs, calves and lambs (Kurtz and Lee, 1987). Feline astroviruses FastV-D1, D2 and D3 were detected in all samples. FastV-D1 was found to be the most closely related to mink and California sea lion astroviruses (58% and 56% identity) in the Cap region, suggesting it is a novel species (Zhang et al., 2014). Coronaviruses and bocaviruses were the next two most prevalent viruses (8.05% and 2.6% of all viral reads, respectively) (Zhang et al., 2014). All coronavirus reads from this study were highly related to previously identified feline coronavirus type I, which makes up 80-90% of natural coronavirus infections in cats (Addie et al., 2003; Kummrow et al., 2005). Feline bocavirus FeBoV3-FBD1 and FeBoV1-FBD2 were found in 83% of the pooled sequenced samples (Zhang et al., 2014). FBD1 is a proposed novel bocavirus strain, as it was found to have 68-76% identity with other feline bocavirus strains (NS1 and VP1 proteins). FBD2 NS1 and VP1 proteins had >96% identity with Hong Kong feline bocaviruses (Zhang et al., 2014).

The RdRp gene is a highly conserved gene in positive-strand RNA viruses (Zhang et al., 2014). Zhang et al. (2014) identified four RdRp sequence contigs (assembled sequences) with low similarity to known sequences, and further analyzed these sequences using the BLASTp. It was found that the first divergent RdRp sequence shared 30% identity with foot-and-mouth disease virus (FMDV), the second shared 30% identity with hepatitis E virus (HEV) RdRp gene (genotypes 1-4), the third with 28% identity with calhevirus-1 RdRp and the fourth with 41% identity with tetnovirus-2 RdRp. FMDV (picornavirus) is a disease of cloven-hoofed animals (including cattle, swine, sheep, goats, and deer) that causes fever and vesicular lesions on the tongue, feet, and snout, and has also been found to be lethal in experimental infections of laboratory mice (Grubman and Baxt, 2004; Salguero et al., 2005). In humans, acute hepatitis E infections manifests as anorexia, fever, malaise, and may result in liver damage (Hughes et al., 2010). Both calhevirus-1 and tetnovirus-2 have been previously identified in human fecal samples, but are predicted to naturally infect insect hosts (Zhang et al., 2014). However, the low identities of the identified RdRp sequences indicate

that actual infection of these divergent viruses may not reflect the infections of the most closely related viruses.

Rotaviruses have been previously identified in various animal hosts (cattle, horses, swine, cats, dogs and humans) and can cause gastroenteritis (Otto et al., 2015; Steyer et al., 2008). Two metagenomic studies conducted by Ng et al. (2014) and Phan et al. (2017) identified novel feline rotaviruses. The novel rotavirus Viseu identified by Ng et al. (2014) was most closely related to group H rotaviruses and group B rotaviruses (53% and <47% identity, respectively), which are associated with diarrhea in humans and swine, at the VP6 gene, but the authors were not able to obtain the full genome and were therefore unable to conclude its phylogenetic relationship to other rotaviruses. The rotavirus I strain *Felis cattus* KY026794 detected by Phan et al. (2017) was most closely related to canine rotavirus I strains KE135 and KE528 based on VP1-4, VP6 and VP7 structural proteins with 25-79% amino acid similarity. These results indicate that *Felis cattus* KY026794 (renamed as RVI/Cat-wt/CAN/Felis catus/2016/G3P2) is a novel divergent feline rotavirus I strain, and the authors suggest that RVI/Cat-wt/CAN/Felis catus is a causative agent of feline diarrhea since no other eukaryotic viral sequences or bacterial and protozoan pathogens were detected in the sample (Phan et al., 2017). It is important to note that sequences detected in fecal samples do not reflect actual infections, and further research should be conducted to provide more conclusive evidence of the role that divergent feline rotaviruses play in causing diarrhea.

Many of the viral infections detected in metagenomic studies appear to have no clinical relevance in the animals tested, but further research is needed to associate the found viruses with various disease states in different animals. Prevalence studies detecting different viruses in different populations of cats (wild, companion animals in homes and shelters) could be correlated to certain health states and would be useful information. Of the viruses found, many can or have the potential to cause disease in felids as well as other mammals. Astroviruses were among the most commonly detected viruses in wild and companion cats, and this virus is thought to cause diarrhea in different species (Zhang et al., 2014). The coronavirus sequences found in some shelter cats all had high sequence identity to previously reported coronaviruses, while 19 sequences with relatively low identity (76%-79%) to known feline herpesvirus type 1 (FHV-1, the causative agent of feline viral rhinotracheitis) were also identified (Zhang et al., 2014). Feline calicivirus was detected in one metagenomic study and this virus has been previously correlated with infections of the upper respiratory tract,

pneumonia and lower urinary tract disease (Stiles, 2014; Zhang et al., 2014). Importantly, viral sequences closely related to human strains of bocaviruses, picorbirnavirus, calhevirus-1, and tetnovirus-2 were detected in the fecal samples of domestic cats, and the authors suggest that these may have resulted from previous human-feline virus transmission, indicating that domestic cats may be a reservoir for multiple zoonotic and potentially zoonotic viruses (Ng et al., 2014; Zhang et al., 2014). These results emphasize the wide range and divergence of fecal viral species that can be found in felids. Further studies are required to better characterize the viral populations in larger populations of wild and domestic cats to understand the role of enteric viruses in causing felid and, possibly, human disease.

1.3.2 Ferrets

Ferrets are popular small companion mammals, and are susceptible to many of the same classes of viruses that can cause disease in humans. It has been shown that infection with human influenza A virus, nipah virus, various morbilliviruses, and severe acute respiratory syndrome (SARS) coronavirus can cause active disease in ferrets (Smits et al., 2013). Ferrets are commonly used as animal models for human viral infections in research, and as such, their viral biome composition is of interest not only for researchers but also because of the zoonotic potential of these viral infections, as humans may also be susceptible to ferret viruses (Smits et al., 2013).

Rectal swab samples from farm, laboratory, and companion ferrets were sequenced in a metagenomic study by Smits et al (2013). Viral sequences with high similarity (85%-100% amino acid identity) to murine astrovirus, ferret coronavirus, ferret hepatitis E virus and Aleutian mink disease were found, as well as the first reports of kobuvirus, parechovirus, papillomavirus, and ferret anellovirus in these rectal samples (Smits et al., 2013). Real-time polymerase-chain reaction (PCR) confirmed that companion ferrets shed ferret hepatitis E virus and kobuvirus more often than farmed ferrets, but coronaviruses were identified in both companion and farmed ferrets. The astrovirus sequences obtained have ~95% identity to a recently described murine astrovirus STL1. The ferret kobuvirus identified in this study, MpKoV38, is most similar on the nucleotide level to bovine kobuvirus BKoV in the P1-3 regions, with 80.0-89.5% identity (Smits et al., 2013). In addition to the known viruses, a 2,207-amino acid polyprotein parechovirus sequence found in a healthy animal is highly divergent from human parechovirus with less than 43% identity in the entire polyprotein (P1-

3), but had similar genome organization. The authors named this new parechovirus MpPeV1, and suggested that its low level of similarity to other parechoviruses could indicate that it is a new genus within the family *Picornaviridae* (Smits et al., 2013). Finally, a papillomavirus (MpPV1) identified from sequencing then amplified and re-sequenced was found to have 60% identity to canine papillomavirus 2. Papillomaviruses are not a common finding in mammalian fecal samples and have not been associated with gastroenteritis, thus, the impact of MpPV1 infection on ferret health is unclear (Mihalov-Kovács and Krisztia, 2014; Smits et al., 2013).

In a metagenomic study on the fecal virome of 18 diarrheic shelter ferrets, Fehér et al. (2014) found high levels of ferret coronaviruses (50% of animals tested) and five different strains of gyrovirus (44% of animals tested), including human strains HGyV and GyV3, and avian strains AGV2 and CAV, as well as GyV4, which has been isolated from human stool and chicken products (Tung G. Phan et al., 2012; Sauvage et al., 2011). Through further PCR amplification and sequencing, the authors found that strains GyV3 and CAV had 99%-100% identity to previously identified gyroviruses (Fehér et al., 2014). It is interesting to note that many of the samples used in this study were collected from ferrets with lymph node and splenic enlargement. Novel gyroviruses have been correlated to diarrheal disease in humans and chickens in other studies (Chu et al., 2012; Phan et al., 2015; Tung G. Phan et al., 2012), and the authors suggest that these new gyroviruses may have different clinical effects in ferrets (Fehér et al., 2014). Although the authors were not able to draw any conclusions on the relationship between gyrovirus shedding and ferret disease, they state that human-ferret transfer of gyroviruses is likely possible due to the high nucleotide similarities between ferret and human gyroviruses (Fehér et al., 2014). Coronaviruses have also been shown to cause disease in several animal species (Fehr and Perlman, 2016), and ferret enteric CoV is known to cause diarrhea, lethargy, anorexia, and vomiting in ferrets (Provacia et al., 2011).

Interestingly, both metagenomic studies identified various avian viruses in both companion and farmed ferrets, including chicken anemia virus (CAV), astrovirus, and parvovirus, turkey hepatitis virus and parvovirus, fowl adenovirus, and avian gyrovirus (Fehér et al., 2014; Smits et al., 2013). The authors of both studies suggest that these may have resulted from the ferrets' poultry-based diet (Fehér et al., 2014; Smits et al., 2013).These studies provide evidence for the diversity of viruses shed in the feces of ferrets, and show that novel and potentially zoonotic viruses are being shed from companion and

farm ferrets. Since ferrets and humans are susceptible to diseases by some of the same viruses, the discovery of novel viruses in ferrets warrants further monitoring to determine their role in ferret and, possibly, human health and disease. These studies also demonstrated the need to further investigate the role of diet in virus shedding, as the authors state that presence of avian viruses was likely a direct result of the ferrets' diet (Fehér et al., 2014; Smits et al., 2013).

1.3.3 Wild carnivores and rodents

Zoonotic agent transmission from wild animals to humans have caused multiple disease outbreaks in which the causative agent was a novel or divergent virus (Bodewes et al., 2014; Huynh et al., 2012; Kreuder Johnson et al., 2015; Li et al., 2005). Wild species generally have increased exposure to other animals and environmental factors, and can therefore be carriers of viruses not commonly found in domestic animals (Bodewes et al., 2014). In fecal virome studies using samples collected from wild animals, the environment and diet of different species and even different animals may vary to a large degree. These inconsistencies could result in significant differences in the viruses present in the samples when subjected to amplification and NGS, but may be valuable in demonstrating the effects of environment and diet on the virome.

Viral studies involving wild American mink, European mink, European polecat, European pine marten, stone marten, Eurasian otter, Eurasian badger, common genet, red fox, Egyptian mongoose, otters, and European wildcat have been conducted to provide an overview of the prevalence of expected known viruses as well as to identify new viral infections (Bodewes et al., 2014; Conceição-Neto et al., 2015; Duarte et al., 2013). Bodewes et al. (2014) evaluated 42 fecal samples from 10 species of small wild carnivores (American mink, European mink, European polecat, European pine marten, stone marten, Eurasian otter and Eurasian badger, common genet, red fox and European wild cat) from Northern Spain using random PCR amplification and NGS. Of the samples tested, sequences from the genera *Circovirus*, *Picobirnavirus*, *Astrovirus*, *Parvovirus*, *Kobuvirus* and *Gammaretrovirus* were detected in most of the samples (Bodewes et al., 2014). Parvoviruses have relatively high prevalence in the Egyptian mongoose, red fox, stone marten, common genet, Eurasian badger, and European mink. Parvovirus sequences detected in the European mink sample had >95% identity to turkey parvovirus, which the authors suggest was likely the result of diet

(Bodewes et al., 2014). Mink calicivirus strain MCV-DL/2007/CN and Aleutian disease-causing parvovirus were also detected in both European and American mink samples with >95% identity (Bodewes et al., 2014). Several novel and divergent viruses were also identified by Bodewes et al. (2014), including theilovirus, phlebovirus, amdovirus, kobuvirus, and picobirnaviruses. Interestingly, a divergent kobuvirus discovered in red fox fecal samples, tentatively called red fox kobuvirus (KF823813), has 97% nucleotide identity with a canine kobuvirus previously detected in Italy (Bodewes et al., 2014). This high level of similarity suggests that transmission of this virus between red foxes and dogs is possible or that interspecies transmission occurred recently (Bodewes et al., 2014). Conceição-Neto et al. (2015) sequenced 3 fecal samples collected from a badger, mongoose, and Eurasian otter. They were able to identify 5 novel gemycircularviruses from the badger and mongoose fecal samples (Mongoose feces-associated gemycircularvirus a-d and Badger feces-associated gemycircularvirus), which all had low similarities to all previously identified gemycircularviruses, and shared 57.2-71.7% identity between each other (Conceição-Neto et al., 2015). A partial RdRp sequence of a novel nodavirus in the otter sample with 43% identity to the RdRp sequence of Mosinovirus, which had been previously isolated from mosquitoes (Conceição-Neto et al., 2015). Duarte et al. (2013) conducted a preliminary study to assess the prevalence of parvovirus, coronavirus, canine distemper virus (CDV), feline herpesvirus, pseudorabies virus, canine adenovirus 1-2 and canine influenza A virus in 34 samples collected from wild Egyptian mongoose, red fox, stone marten, common genet and Eurasian badger found dead (presumed road kill) in Portugal. Parvoviruses were found in 63% of all samples, while all samples were negative for CDV, feline herpesvirus, pseudorabies virus, canine adenovirus 1 and 2, and influenza A virus (Duarte et al., 2013). Further PCR testing in 128 specimens determined that parvovirus DNA was detected in 63.3% of samples indicating that parvovirus exposure in wild Egyptian mongoose, red fox, stone marten, common genet and Eurasian badger is common in Portugal (Duarte et al., 2013).

Rodents are present in high densities in both urban and rural areas, and can come into contact with various wild and domestic animals, as well as with humans. Rodents have been known to host many pathogenic and zoonotic viruses, such as the Sin Nombre virus (SNV), which causes Hantavirus pulmonary syndrome (HPS), Tula virus, which is associated with acute infection, and Lassa virus, which causes Lassa fever (Phan et al., 2011). As such, the viromes of wild rodents are of interest due to past successful zoonotic events that have caused a range of sporadic to enzootic diseases.

Phan et al. (2011) sequenced the fecal viromes of 105 wild rodents, including mice, voles and rats, and found that 52% (of 28,846 sequences) of the virus-related sequences were mammalian viruses, with most related to DNA viruses. Since the samples were collected from wild rodents, the fecal matter may have been exposed to a variety of different viruses from various plants, fungi and bacteria (Phan et al., 2011). The most abundant of the mammalian viruses were from the family *Circoviridae* (90% of mammalian DNA virus-like sequence reads), and viruses from the families *Parvoviridae, Papillomaviridae, Astroviridae* and *Adenoviridae* (Phan et al., 2011). A new papillomavirus, PmPV1, in the *Iotapapillomavirus* genus, was found to be most closely related to rodent papillomavirus MnPV1 in the L1 region (capsid proteins) with 67% identity (Phan et al., 2011). The authors reported the first case of a kobuvirus in mice (*Peromyscus crinitus* and *P. maniculatus*), which is interesting since kobuviruses are associated with acute gastroenteritis in numerous species, such as pigs, cows, and humans (Phan et al., 2011). Adenoviruses (AdV), which can cause respiratory diseases and acute gastroenteritis, were also identified in small fragments, along with small adeno-associated viruses (AAVs) that have not been directly associated with any diseases in humans (Phan et al., 2011). The AdV sequences identified encoded 10% of the AdV hexon protein (major coat protein) and was most closely related to murine AdV2 with 80% identity based on the identified hexon protein sequences (Phan et al., 2011). AAV sequence reads covered 40% of the AAV VP7 protein (outer capsid protein) and showed highest similarity to porcine AAV at 61% identity based on the identified VP7 sequences (Phan et al., 2011). Additionally, Phan et al. (2011) detected mosavirus (mouse-stool-associated-picornavirus) and rosavirus (rodent-stool-associated-picornavirus) sequences, which were further genetically characterized. The detected rosavirus is distantly related to kobuviruses, but had <31% similarity at the P3 region (encodes polymerase) to the closest reported picornaviruses, while the Mosavirus was distantly related to cardioviruses, another form of picornavirus, but had <40% similarity to other reported picornaviruses (Phan et al., 2011). Rosavirus and mosavirus had only 23% similarity to each other, and because both viruses showed a high level of divergence from other picornaviruses, the authors proposed that these could belong to two new genera in *Picornaviridae* (Phan et al., 2011). Both urban and rural rats were found to be seropositive for anti-HEV IgG, but prevalence was higher in urban rodents (Meng, 2010; Pavio et al., 2010). This is significant as it could mean that urban rodents may act as a reservoir for HEV and have a role in the transmission of the virus,

although it is still unclear whether the genetically distinct rat HEV can infect humans (Meng, 2010; Pavio et al., 2010).

Monitoring of wild carnivore and rodent fecal viromes allows identification of novel or divergent viruses with zoonotic potential, which may help to control future transmissions. Geographic mapping of animals and their associated infections in different regions may be useful epidemiologically to identify potential sources of transmission and determine exposure levels between urban and rural areas. Comparative sequencing between carnivores, whether wild, commercial or domestic, has demonstrated that a highly diverse range of known mammalian, plant and insect viruses as well as many novel viruses of interest can be found in fecal samples (Bodewes et al., 2014; Conceição-Neto et al., 2015; Duarte et al., 2013; Phan et al., 2011). It is important to note that viral sequences detected in the feces of these animals may not reflect active viral infections or the ability of these viruses to be transmitted to other animals (Pinsky et al., 2010). The zoonotic potential and pathogenicity of these viruses in different species still need to be assessed, but these studies have demonstrated that metagenomic virome characterization is highly useful in expanding our knowledge of virus shedding by different species as well as the prevalence of viruses in different animal populations.

1.4 Viral Diseases of Commercial Mink

There are several viral diseases of commercial mink that may cause significant economic losses, including Aleutian disease (AD), canine distemper, influenza A, and viral enteritis. Commercial mink are usually vaccinated against distemper, viral enteritis, *Pseudomonas* pneumonia, and botulism in a single 4-way vaccine by 12 weeks of age (Newman et al., 2002; Rikula et al., 2001). It is important to note that although the use of this vaccine is common on farms in Ontario (Compo et al., 2017), the code of practice for farmed mink does not require vaccination against specific pathogens (NFACC, 2013).

1.4.1 Aleutian disease

First reported in the USA in the late 1940s (Gorham et al., 1965), Aleutian disease is caused by a parvovirus (ADV). It is classified under the genus *Amdoparvovirus* (family *Parvovirindae*) as carnivore amdoparvivirus-1, and is a naked virus of 22-26 nm diameter, characterized by two proteins, VP1 and VP2 (minor and major capsid proteins) (Bloom et al.,

2001; Nituch et al., 2012). The viral capsid contains a single strand of DNA and is ~4.8 kb in size (Bloom et al., 2001). ADV is readily transmitted through mink body fluids, such as urine, feces, blood, and saliva, and is transmitted in utero across the placenta. The virus is able to remain intact under various environmental pressures, surviving high temperatures and chemical treatments, and decontamination and eradication are difficult (Porter et al., 1977; Prieto et al., 2014).

In mink, AD can result in poor reproduction, rapid weight loss, oral and gastrointestinal bleeding, renal failure, and often is associated with high mortality (Jepsen et al., 2009). ADV infection may result in immune system dysregulation, plasma cell infiltration into the kidneys, liver, spleen, lymph nodes, and bone marrow, as well as bile ductule proliferation (Jepsen et al., 2009). In adult mink, ADV infections are usually chronic, characterized by high anti-ADV antibody levels, segmental or circumferential arteritis with fibrinoid necrosis of the tunica muscularis, and hypergammaglobulinemia (Alexandersen et al., 1994; Jepsen et al., 2009). In kits, ADV causes an acute cytopathic infection of alveolar type II pneumocytes, characterized by low levels of anti-ADV antibodies and pneumonia, followed by death within 3 weeks of initial infection (Alexandersen et al., 1994; Jepsen et al., 2009). It has also been established that mink with sapphire and pearl coat colours have a much higher mortality rate compared to dark, wild-type mink, and the severity of disease is also dependent on the strain of virus (Bloom et al., 1975; Budd et al., 1966; Wiedbrauk et al., 1986). Even low virulence strains of ADV can cause higher mortality rates in sapphire mink, but only cause mild disease with low mortality in non-Aleutian mink (Bloom et al., 1975; Wiedbrauk et al., 1986). In addition to lower production efficiency, AD has also been associated with undesirable white tail hairs, which reduces the value of pelts (Farid and Ferns, 2011).

AD is thought to have started with the rise of mink farming in North America in the late 1800s, and it is still unclear whether the virus originated from a wild source or if it evolved independently within the commercial mink populations (Nituch et al., 2012). It is thought that AD was first brought to Europe with the introduction of mink fur farming in the 1920s (MacDonald and Harrington, 2003). In eastern Canada, ADV is highly prevalent in wild mink (~93% are positive for the ADV or anti-ADV antibodies) (Farid, 2013). It has also been detected in a wide variety of other wild animals in Nova Scotia, including weasels, striped skunks, North American river otters, raccoons, and bobcats (10% to 70.5% prevalence

levels) (Farid, 2013). Mink farmers have also tested positive for serum antibodies to ADV, and upon post-mortem evaluation of medical history and autopsy results, it was found that the farmers had some abnormalities similar to the disease presented in ADV infected mink, including chronic glomerulonephritis, mesangial hypercellularity, decreased kidney size, stenosis of the renal, mesenteric and axillary arteries, and lymph node T-zone hyperplasia (Jepsen et al., 2009). A study conducted in 2006 found that the prevalence of AD on mink farms in Nova Scotia was 33.3% (n=27), although this study is limited in the low number of sample submissions and therefore may not be representative of true provincial prevalence (Newman and Reed, 2006). Between the years 1986 and 2006, other studies conducted in Ontario have reported prevalence of AD-positive reactors in sampled mink populations to be between 14-60%, although farm participation was low for these surveys as well (Nituch et al., 2011). It is important to note that a study conducted by Preito et al. (2014) on Spanish mink farms found that 93.9% of environmental samples (silo, soil from truck ruts, two samples from effluents, workers' outdoor footwear and street clothes, warehouse, cage walls and a sample from each of the two cars belonging to the farms) were positive for ADV DNA in qPCR analysis.

So not only does AD present a major production challenge for commercial mink farmers, it also has the potential for widespread infection to wild species, as well as zoonotic transfer to producers and other humans working frequently on infected or previously infected farms (Farid, 2013; Jepsen et al., 2009). More comprehensive prevalence testing, sequencing, and disease studies are needed to better understand virus epidemiology.

1.4.2 Canine distemper virus

Canine distemper virus, which belongs to the genus *Morbillivirus* within *Paramyxoviridae*, is an enveloped virus that is 150-300 nm in diameter, with linear, negative sense single stranded RNA (ssRNA) (~15.7 kb) (Trebbien et al., 2014). Characteristic viral proteins include matrix (M), fusion (F), hemagglutinin (H), nucleocapsid (N), polymerase (L), and phosphoprotein (P), where the highly variable H protein is required to attach to host cells (Trebbien et al., 2014). The virus induces disease in dogs, foxes, wolves, mink, ferrets, badgers and martens (Gregers-Jensen et al., 2015). Vertical transmission may have allowed for the continued persistence of the virus, but transmission occurs mainly when the virus is

aerosolized (Trebbien et al., 2014). CDV can be inactivated through treatment with UV-light, higher temperatures and drying (Gregers-Jensen et al., 2015).

CDV can cause disease in mink of all ages. Clinical presentation is characterized by crusting at the eyes and nostrils, and diffuse thickening of the footpads (Budd et al., 1966; Dyer and Schamber, 1999). Some infected animals may develop conjunctivitis and fever, along with viremia, lymphopenia and decreased lymphocyte proliferation, as well as an enlarged spleen (Budd et al., 1966; Zhao et al., 2015). Convulsions and frothing at the mouth may also be presented, but affected animals will eat until unable to reach their food (Budd et al., 1966). Histology of affected tissues (for example, epithelial cells of the bladder, kidney, respiratory tract, and intestine) from diseased mink may demonstrate intracytoplasmic and intranuclear eosinophilic inclusion bodies (Budd et al., 1966; Green and Evans, 1939).

Implementation of various on-farm biosecurity measures may help to avoid introduction of the virus, but biosecurity on most Ontario farms is often low (Compo et al., 2017; Gregers-Jensen et al., 2015). There is no treatment for canine distemper, and only vaccination prior to infection is effective. As vertical transmission of CDV can occur, breeding females are re-vaccinated in January or February to prevent infection of kits (Budd et al., 1966).

1.4.3 Influenza A

Mink influenza A virus is from the genus *Influenza A* virus within the family of *Orthomyxoviridae*. Most natural influenza virus infections do not cause disease in mink, but a new subtype, H3N2, was found to cause disease in commercial mink in 2006 (Gagnon et al., 2009). Sequencing results of this virus demonstrated that the H and N genes were most similar to the human H3N2 virus. This subtype H3N2 is not only a reassortant of the human and swine influenza A virus, but is also closely related to the pandemic H1N1 virus (Gagnon et al., 2009). Infection in mink is characterized by respiratory disease in adults and kits, and co-infection with other pathogens may increase mortality. Clinical signs include lethargy, oronasal hemorrhage, and catarrhal to watery discharge from the eyes (Gagnon et al., 2009). There are many potential sources of H3N2 infection that are not mink specific, including any raw pork and poultry products commonly used in the mink diet, and infected human caregivers (Gagnon et al., 2009).

A study conducted to assess the prevalence of H9N2 in Chinese commercial mink found that 45.4% (254/560) of samples were seropositive for Ck/HB/4/08 (H9N2), 47.5% (266/560) were seropositive for A/Chicken/Shanghai/10/01 (H9N2), and 6.4% (36/560) were seropositive for H5N1 (RE-7) (Zhang et al., 2015). Both young and adult mink had high serum positivity against two H9 antigens (A/chicken/Shanghai/10/01 and A/Chicken/Heibei/4/2008) (Zhang et al., 2015). These studies show that mink are susceptible to both mammalian and avian influenza viruses, and further monitoring of influenza viruses in different mink populations is needed provide more information on the prevalence of these zoonotic viruses.

1.4.4 Mink viral enteritis

Mink enteritis virus (MEV) is a parvovirus from the family *Parvoviridae*. It is a spherical virus that is 18-26 nm in diameter, containing ssDNA that is 5094 nt in genome size and contains two major open reading frames (ORF) (Kariatsumari et al., 1991). One 3' half ORF encodes NS1 and NS2 non-structural proteins, while the 5' half ORF encodes the capsid VP1 and VP2 proteins (Wang et al., 2015). Since the first report of the disease in 1949, MEV has caused serious financial losses in the global mink fur industry (Schofield, 1949).

MEV can infect both adults and kits, and signs generally begin to develop within a week of initial infection (Arnold et al., 1997). Mink viral enteritis is characterized by anorexia, watery, mucoid or bloody diarrhea leading to dehydration and death, although sudden anorexia followed by death without diarrhea has also been recorded (Reynolds, 1969). Some animals have died without gross lesions, but most have intestinal and splenic and lymph node lymphoid depletion. Mink may survive, although are generally unthrifty (Reynolds, 1969).

To prevent disease, all animals are vaccinated at 6-8 weeks of age. In the case of an outbreak, all mink showing clinical signs need to be isolated or culled, and the healthy animals vaccinated (Reynolds, 1969). A nanoPCR assay has been developed to more effectively detect MEV in domestic mink, as prior methods were either too time consuming and expensive or lacked specificity (Wang et al., 2015).

1.4.5 Shaking mink syndrome

Shaking mink syndrome (SMS) is a neurologic disease of low incidence characterized by shaking (slight tremors to seizures), staggering, salivation, screaming, ataxia (Blomström et al., 2010). Kits provided with ready access to feed and water are more likely to survive, and affected mink at the terminal stages are often unable to move and sensitive to noise (Gavier-Widén et al., 2004). SMS was first reported in 2000 mink kits on a farm in Denmark, with the highest incidence rates observed in June and July (Blomström et al., 2010; Gavier-Widén et al., 2004). Gavier-Widén et al. (2004) found morbidity on 13 affected farms in Sweden and Denmark to be approximately 0.2% (229/35,253) and 0.8% (265/13,0671) respectively. The mortality rates were 28% (n=296) on Swedish farms and 27% (n=265) on Danish farms (Gavier-Widén et al., 2004). Blomström et al. (2010) determined that SMS is associated with a specific astrovirus infection, SMS-AstV, which was isolated from the brains of affected kits. SMS-AstV has 98% similarity to a diarrhea-inducing mink astrovirus (MiAstV) at the ORF1 and ORF1b genes, but only 67% amino acid similarity at the ORF2 (capsid protein) gene (Blomström et al., 2010). Although SMS-AstV grouped closely with MiAstV and an ovine mink astrovirus (OAstV-1), it was determined to be a different phylogenetic clade (Blomström et al., 2010). Blomström et al. (2010) detected no other viruses using sequencing methods, which further supports the correlation between SMS-AstV infection and the development of the neurological signs of disease typically seen in SMS.

1.4.6 Catarrhal gastroenteritis

Epizootic catarrhal gastroenteritis (ECG) in mink is caused by mink *Alphacoronaviruses* (MCoVs), within the subfamily of *Coronovirinae* (Vlasova et al., 2011). The virions vary greatly in size (60-220 nm) and shape, with a crown-like exterior formed by the surface spike glycoprotein (Vlasova et al., 2011). ECG results in anorexia and mucoid diarrhea within a week of infection, but is usually self-limiting and clinical signs rarely exceed a week (Gorham et al., 1990). Infection is associated with almost 100% morbidity, but less than 5% mortality, with higher incidence of disease observed in darker furred mink >4 months of age (Vlasova et al., 2011; Gorham et al., 1990). Mortality rates may be higher in mink co-infected with ADV due to immune system suppression (Gorham et al., 1990). Mortality is also higher if ECG occurs during the nursing period since kits may go off feed, but the disease usually occurs in early fall up to the time of pelting, long after weaning has occurred (Gorham et al., 1990). Between 1975-1989, Gorham et al. (1990) estimate that

several million mink have been affected in North America, Scandinavia, the regions formerly part of USSR and China. However, a study conducted by Wilson et al. (2015) found that between 2009-2014, only 4.1% of 339 mink post mortem cases (submitted from farms in western USA) have been caused by ECG. This further supports that although morbidity caused by ECG is high, mortality is generally low.

1.5 Other enteric viruses

Mink-specific astrovirus, rotavirus and hepatitis E virus (HEV) have been associated with various enteric and respiratory diseases in commercial mink, and have been shown to cause kit mortality in viral outbreaks or are associated with disease in other species (Mittelholzer et al., 2003; Otto et al., 2015; Pavio et al., 2010; Vu et al., 2017). Although correlations have been drawn between these three viruses and disease, it has been suggested that other co-factors, mainly other viruses, may be at play (Gorham et al., 1990). Focusing on identifying and characterizing these viruses may help to better understand disease susceptibility as well as providing information on possible interventions in controlling outbreaks. Importantly, these three viruses have also been known to cause both enteric and respiratory disease in other mammalian species, including humans, and therefore there is a need to assess the commercial mink population as a reservoir for potentially zoonotic viruses (Mittelholzer et al., 2003; Otto et al., 2015; Vu et al., 2017). To date, the prevalence of these three viruses is unknown in commercial mink and there are no diagnostic laboratories that routinely test samples for these viruses in Canada.

1.5.1 Enteric astrovirus infections

Astroviruses are icosahedral non-enveloped, positive sense, ssRNA viruses that are ~28 nm in diameter with a genome size of 6.4-7.3 kb (Finkbeiner et al., 2009; Vu et al., 2017). The three ORFs, ORF1a, ORF1b and ORF2 are followed by a poly A tail at the 3' end, where ORF1a is thought to encode viral protease (non-structural protein), ORF1b encodes RdRp and ORF2 encodes non-structural proteins (Blomström et al., 2010). Astroviruses have been associated with enteric diseases in many species, including birds, wild animals and humans (De Benedictis et al., 2011; Mendenhall et al., 2015). Studies sequencing astrovirus RNA from animal and human samples have shown great genetic diversity suggesting frequent recombination events (De Benedictis et al., 2011). Mink astrovirus (MiAstV) is considered to play an important role in causing pre-weaning diarrhea, and is highly prevalent

on farms in Denmark (De Benedictis et al., 2011; Englund et al., 2002; Mittelholzer et al., 2003). MiAstV is phylogenetically grouped with human astroviruses (*Mamastroviruses*), although not closely related (Vu et al., 2017). MiAstV shares only 67% identity with the closest related ovine astrovirus, OAstVA (De Benedictis et al., 2011). A human-mink-ovine-like astrovirus (VA/HMO) has been identified in the fecal sample of a human with diarrhea (Vu et al., 2017). Astroviruses seem to be highly species-specific (Mittelholzer et al., 2003), and although cross-species transmission would be rare, sustained opportunities for exposure, as in the case with producers of infected animals, increase the risk of transmission to incidental hosts (Mendenhall et al., 2015).

1.5.2 Rotavirus infections

The rotavirus genome is composed of 11 segments of double stranded RNA (dsRNA), which encode six structural viral proteins and six non-structural viral proteins (Otto et al., 2015). The G3 RVA genotype of rotavirus has been found in samples from humans and other mammalian species as well as avian species, and is known to cause infantile diarrhea in humans and these animals (Bonica et al., 2015; Steyer et al., 2008). There is strong evidence of interspecies transmission, as a rotavirus isolated from rabbit was closely related to bovine-like rotavirus strains, and in a different study, a rabbit rotavirus was able to cause disease in a child (Bonica et al., 2015; Schoondermark-van de Ven et al., 2013). Rotavirus-like particles have been detected in the fecal matter of mink, but rotavirus has not been directly correlated with enteric disease and is thought to be a co-factor in mink enteritis, and the zoonotic potential of the detected rotavirus has not been assessed (Gorham et al., 1990). Unlike astroviruses, rotaviruses are considered to be zoonotic agents, and reassortment of the segmented genome is not uncommon (Steyer et al., 2008). The risk of zoonotic transmission of porcine and bovine rotaviruses is higher on production farms (Steyer et al., 2008). Currently, there are no reports of rotavirus in mink in Canada.

1.5.3 Hepatitis E virus (HEV) infections

Hepatitis E virus (HEV) is a positive sense, ssRNA virus in the *Hepeviridae* family that is approximately 33 nm in diameter, with a genome 7.2 kb in size with three ORFs (Meng, 2010; Pavio et al., 2010). The genome of HEV is capped at the 5’ end and has a polyadenylated tail at the 3’ end (Pavio et al., 2010). ORF1, which takes up about two thirds of the entire viral genome, encodes non-structural proteins such as helicase, RdRp and

methyltransferase (Pavio et al., 2010). ORF2 encodes the viral capsid protein and ORF3 encodes a phosphoprotein. The *Hepeviridae* family has four major genotypes, where genotypes 1 and 2 are found exclusively in humans and have resulted in major epidemics, especially in developing countries where sanitation is poor (Pavio et al., 2010). Genotypes 3 and 4 can be found in both humans and other species (Pavio et al., 2010). HEV has not been associated with clinical infections in other mammalian species with the exception of humans, where HEV infection can result in acute liver disease, potentially leading to acute liver failure in pregnant women or chronic infections resulting in cirrhosis in immunocompromised individuals (Pavio et al., 2010). There is experimental evidence that zoonotic transfer of HEV within genotypes 3 and 4 is possible between humans and other animals (Meng, 2010). A mink HEV variant has been detected in four farmed mink fecal samples from Denmark, which shared approximately 65% identity to classical HEV genotypes 3 and 4, and 76% and 69% identity to ferret and rat HEVs respectively (Krog et al., 2013). Commercial swine are known to be a reservoir of HEV with zoonotic potential, and mink are often fed a mixed diet, which may include undercooked pork products (Kasorndorkbua et al., 2004; Krog et al., 2013; Pavio et al., 2010). Krog et al. (2013) suggest that diet is an important source of infection for farmed mink, which is consistent with previous findings that demonstrated the transmission of influenza A virus (H1N2) from undercooked turkey products to mink (Yoon et al., 2012). The mink HEV positive fecal samples tested by Krog et al. (2013) were collected from herds affected by viral enteritis, catarrhal enteritis, Aleutian disease, and hepatic lipidosis, and although HEV could not be directly correlated to any of these disease states, the authors state that mink HEV variant could contribute to clinical signs (Krog et al., 2013).

1.6 Antimicrobial-resistance genes (ARG) in viruses and the mink microbiome

Antimicrobials are commonly used in commercial livestock and poultry to increase growth rates and to treat infectious disease. The overuse of antimicrobials results in selective pressure on bacterial communities that may result in resistance to antimicrobials. Antimicrobials may also leak into ground water and aquifers through manure runoff, creating widespread antimicrobial resistance risks to humans, wildlife, and various domestic animals (Balcazar, 2014; Colomer-Lluch et al., 2011b; Ross and Topp, 2015).

1.6.1 Antimicrobial resistance genes in phage

Phages are viruses with bacterial hosts, and have recently been gaining attention as a potential reservoir of ARGs which may be transferred to bacterial populations (Allen et al., 2011). ARGs are primarily transferred to bacteria through plasmids or integrative and conjugative elements (mobile genetic elements) (Colomer-Lluch et al., 2011a). The transfer of ARGs through phages is not as well understood as other vectors, but some reports have suggested that horizontal transfer via phage may play an important role in the spread of ARGs (Colomer-Lluch et al., 2011b; Volkova et al., 2014). Studies have demonstrated that the number of detectable ARGs in the phage metagenome increases after antimicrobial treatment of animals (as evidenced in mink, mice and dairy cows), which may serve to protect their bacterial hosts, allowing for an increased active phage population even when bacterial populations are under antimicrobial stress (Modi et al., 2014; Pedersen et al., 2009; Ross and Topp, 2015). Metagenomic studies identifying ARGs in phages from a variety of sources have reported a high number of phage-encoded ARGs; however, these studies have been predominantly exploratory in nature (Colomer-Lluch et al., 2011; Fancello et al., 2014; Rolain et al., 2011). A recent study investigating the identification of phage-encoded ARGs in metagenomic virome studies concluded that most viral metagenomic studies have grossly overestimated the number of ARGs detected in phage sequences (Enault et al., 2017). The authors concluded that there is little selective pressure for phages to carry ARGs, and that the transfer of ARGs from phage to bacterial species is a naturally rare occurrence (Enault et al., 2017). Consistent with this conclusion, many sequenced clones of identified ARG-like genes from metagenomic studies have not conferred the predicted antimicrobial resistance to the associated bacterial species (Balcazar, 2014; Enault et al., 2017). Reports of high ARG frequencies may be due to less stringent exploratory search methods, which the authors have defined as poor coverage of target ARG (<40%), low identity (<70%), and low bit-scores (<70%) (Enault et al., 2017; Gibson et al., 2015). Additionally, bacterial contamination may not have been adequately controlled for in these studies (Enault et al., 2017). Using more stringent methods for identification, such as >80% identity over >85% coverage of the target sequence, with bit-score >70% may help to reduce the number of false ARG hits in metagenomic studies (Enault et al., 2017; Gibson et al., 2015). In addition to these more conservative parameters, manual screening is recommended to exclude those hits with homology to phage enzymes that serve non-ARG functions, such as dihydrofolate reductase,

which could potentially confer trimethoprim resistance but is most likely involved in nucleotide metabolism (Asare et al., 2015; Enault et al., 2017).

Although transduction of ARGs from phage to bacteria under natural conditions may be less frequent than previously described, phages still have the ability to confer AMR to bacteria. Further research is needed to determine the rate of ARG transduction from phage to bacteria under natural conditions. From a metagenomic perspective, accurate reporting of ARG hits in phage sequences will require more stringent search parameters in combination with manual exclusions of hits that do not function as ARGs in phages. A more representative report of naturally occurring ARG in phages will allow improved risk evaluation of phage-induced resistance and determine the need for intervention at the phage level.

1.6.2 Antimicrobial resistance in commercial mink

In the mink industry, antimicrobials are used to treat diseased animals and limit the spread of infectious agents to other animals, but may also be used as preventative interventions for urinary tract infections (Schwarz, 1994). The most common cases that require the use of antimicrobials are nonspecific diarrheal and respiratory infections, which may result in animal mortality, with estimated peak antimicrobial use in May and November (Jensen et al., 2016). Antimicrobial contaminated feed may also lead to the unintentional spread of antimicrobials to animals on multiple farms in the same geographic region, as Ontario farms within close geographic proximity often source mink feed from the same producer (Enault et al., 2017; Jensen et al., 2016). Based on anecdotal evidence provided through the Ontario Animal Health Network (OAHN), the use of antimicrobials is not well tracked in mink, and historically, producers have obtained antimicrobials without close veterinary supervision. Producers rarely seek veterinary expertise for found dead animals, and often choose to treat animals empirically. Antimicrobial resistance (AMR) in mink following use of antimicrobials has been documented (Pedersen et al., 2009). Evaluation of ARGs from bacterial isolates obtained from antimicrobial-treated mink have reported resistance to tetracyclines, penicillin, lincosamides, chloramphenicol, macrolides and spectinomycin (Pedersen et al., 2009; Schwarz, 1994). It is important to note that biosecurity on mink farms is poor to non-existent (Compo et al., 2017), and the open shed housing systems that are in use in Canada allow for potential interactions between mink and other domestic and wild animal species. Poor personnel hygiene standards on mink farms may also

increase the risk of pathogen transfer to producers. To date, there is little research on antimicrobial resistance in Ontario commercial mink, and no information on the possible contribution of phage in transferring ARGs to bacterial species in the mink gastrointestinal tract.

1.7 Study Rationale

Although the Canadian mink industry has set standards for farm biosecurity and animal husbandry, few farms consistently implement all recommended practices (CFIA, 2013; Compo et al., 2017; NFACC, 2013). The results of this study will characterize the diversity of the viral population in commercial mink, and may serve to emphasize how improved biosecurity guidelines may help to reduce the risk of viral outbreaks that may result in mink mortality, as well as reducing the risk of mink-human virus transmission. This study provides an overview of the prevalence of fecal shedding of three specific viruses, including astrovirus, rotavirus, and HEV on Ontario mink farms, where viral shedding will be compared over four years (2014, 2015, 2016, and 2017). In addition, virus shedding in samples from females and kits from 2014 will be closely analyzed using next-generation sequencing. Sequencing results will provide more information on viral diversity in the Canadian farmed mink population and help to identify divergent viruses, both mammalian and bacterial in nature. Phage sequences will be screened for known antimicrobial resistance genes. Overall, the results of this study will allow comparisons to be drawn between adult female mink and mink kits, farms, and other environmental factors, all of which affect exposure to different viruses, and therefore, animal health. Results from direct antimicrobial testing from bacterial isolates will be compared to antimicrobial resistance genes detected in phage sequences, which will allow determination of correlations between phage-encoded antimicrobial-resistance genes and bacterial antimicrobial resistance.

1.8 Study Objectives and Hypotheses

1.8.1 Study objectives and methods

There are four main objectives of the current study:

1. To determine and compare the prevalence of astrovirus, rotavirus and HEV shedding in pooled fecal samples from adult female mink and mink kits on 43 Ontario mink

farms over four years (2014, 2015, 2016, 2017) using virus-specific primers to amplify targeted regions by PCR.

2. To amplify, sequence, and identify the most prevalent mammalian and bacterial viral sequences (i.e., top hits) shed in pooled fecal samples collected from 2014.
3. To compare differences in viral species identified between adult female mink and mink kits as well as between Ontario farms.
4. To compare sequences of top viral hits to previously reported viruses to compare sequence identity.
5. To identify phage-encoded ARGs and compare to results from antimicrobial resistance testing of bacterial isolates.

1.8.2 Study Hypotheses

1. Astrovirus will be the most prevalent of the three viruses tested in the virus-specific polymerase-chain reactions (PCRs), as it has been previously found to be highly prevalent on mink farms. We also expect hepatitis E virus to be detected, in a moderate number of samples. Since our samples are collected from clinically healthy mink, we do not expect to find an abundance of rotavirus infections.
2. Mink kits will demonstrate more fecal virus shedding than females, and will demonstrate more viral co-infections, as we hypothesize kit immune systems are not fully developed making them more susceptible to infections.
3. There will be no significant differences in astrovirus, rotavirus and hepatitis E virus shedding in adult female mink between the four years sampled (2014, 2015, 2016, 2017), since biosecurity and diet do not vary greatly on individual mink farms between years.
4. We expect to see increased virus shedding in summer samples compared to winter samples, since it has been shown that heat-stressed mink (due to thick pelt) makes the animals more susceptible to infections.
5. A diverse population of previously identified mammalian viruses, including parvoviruses, astroviruses, bocaviruses and gyroviruses, with a high proportion of these viruses being avian-associated, will be detected through next-generation sequencing of 2014 fecal samples, consistent with previous findings of viral metagenomic studies in carnivore species.

6. Highly divergent and novel viruses will be identified from next-generation sequencing results, most likely from the families *Astroviridae*, *Parvoviridae* and *Picobirnaviridae*, consistent with previous findings of viral metagenomic studies in carnivore species.
7. Most antimicrobial-resistance gene (ARG) hits will not be functional ARGs that can be transferred to bacterial species, but β-lactamase-related ARGs will be most prevalent, which would be consistent with previous findings in identifying phage encoded ARGs.

Chapter 2: Prevalence of astrovirus, rotavirus and hepatitis E virus shedding in Ontario farmed mink (*Neovison vison*)

Mink astrovirus is known to play a major role in mink pre-weaning diarrhea, and rotavirus and hepatitis E virus (HEV) are considered significant zoonotic agents. These viruses are not monitored in commercial mink, and the role of HEV in mink health is not well understood. Further, rotaviruses have never been reported in Canadian commercial mink, but based on evidence from previous research, rotaviruses could be an important cause of mink diarrhea (Gorham et al., 1990; Otto et al., 2015). This study assessed the prevalence of astrovirus, rotavirus and HEV in 527 pooled adult female mink and mink kit fecal samples from 50 Ontario mink farms in two seasons over four years. Viral RNA was extracted and amplified in RT-PCR. At least one positive sample for each detected virus was sequenced for phylogenetic analysis. For astrovirus, 14% of samples were positive, while 3% and 9% of samples were rotavirus and HEV positive, respectively. A significantly higher number of kit samples were positive for astrovirus and HEV compared to adult female samples ($p=0.01$ and $p<0.0001$, respectively). Rotavirus was detected in significantly more samples from 2017 compared to the 2016 samples ($p=0.05$). Astrovirus RNA was detected in significantly more summer fecal samples compared to winter samples ($p=0.001$). The detected sequences were closely related to previously reported mink-specific astrovirus or HEV strains, and porcine rotavirus strains. Two sequences (mink AV 2017-ON-11az and mink RV 2015-ON-16e) were distantly related to all sequenced mink astrovirus RdRp genes as well as previously reported MiAstV sequences. These results demonstrate low to modest prevalence of the three viruses in feces from clinically healthy Canadian commercial mink, and suggest that further monitoring of these viruses may provide a better understanding of these potentially zoonotic agents and the viral infections that may play a role in mink enteritis.

2.1 Introduction

Astrovirus, rotavirus and hepatitis E virus (HEV) have been associated with enteric and respiratory diseases in a variety of species, including commercial animals and humans. Recent studies found that these viruses are widespread in both domestic and wild animals, and many highly divergent or novel strains have been identified to be zoonotic agents (Krog et al., 2013; Meng, 2011; Mykytczuk et al., 2017; Naccache et al., 2015; Pavio et al., 2010). Recently, human HEV infections from the consumption of contaminated, undercooked pork

and rabbit products have become a significant public health concern internationally, demonstrating the need to investigate and characterize potentially zoonotic viruses in commercial animals (Abravanel et al., 2017; Martin-Latil et al., 2016; Mykytczuk et al., 2017).

In 2015, the Canadian mink industry produced over 3 million pelts, valued at $98 million (Statistics Canada Table 003-0015 2016; Statistics Canada Table 003-0014 2016). Outbreaks of enteritis resulting in kit mortality cause significant economic loss to producers (Compo et al., 2017), and while mink enteritis is most likely the result of multiple viral and bacterial infections, the major causative agents have not been well characterized. Monitoring of viral shedding in healthy and diseased commercial mink may help to identify agents that may play a role in enteric disease, and also permit assessment of the risk of transmission to humans in close contact with these animals. The prevalence of astrovirus, rotavirus and HEV in Canadian commercial mink has not been reported. Mink astrovirus has been demonstrated to play a major role in mink pre-weaning diarrhea (De Benedictis et al., 2011; Englund et al., 2002; Mittelholzer et al., 2003). Both rotavirus and HEV are considered significant zoonotic agents, and are known to cause disease in humans, swine, chicken and rabbits (Krog et al., 2013; Pavio et al., 2010; Schoondermark-van de Ven et al., 2013; Steyer et al., 2008). Both mink HEV and rotavirus-like viral particles have been detected in commercial mink, but neither have been previously directly associated with disease in mink (Krog et al., 2013; Steyer et al., 2008).

The objectives of this study were to evaluate the prevalence and persistence of fecal shedding of mink astrovirus, HEV, and rotavirus on Canadian commercial mink farms. We hypothesized that kits would have higher number of infections for all three viruses than adult female mink (Englund et al., 2002; Mittelholzer et al., 2003). We also hypothesized that viral RNA would be detected in more samples collected in the summer, which has been shown to be true for Gram-negative bacteria in other commercial animals and humans but has not been shown to be true for astrovirus, rotavirus and HEV in mink (Eber et al., 2011; Leekha et al., 2012; Zhang et al., 2016). Additionally, based on other studies, we expected that astrovirus and HEV RNA would be more commonly detected in samples compared to rotavirus RNA (Englund et al., 2002; Krog et al., 2013; Mittelholzer et al., 2003).

2.2 Materials and methods

2.2.1 Sample collection

A total of 527 pooled fecal samples were collected from commercial mink farms across Ontario. In 2014, samples were collected from 43 farms, representing >93% of Ontario mink farms. A total of 50 individual farms were sampled; 43 in 2014, 45 in 2015, 43 in 2016 and 37 in 2017. Over the four-year study period, some farms pelted out, new farms were sampled, some did not participate in subsequent years or only participated in subsequent years. Fecal samples were collected in the summer season (July - October) from adult female mink (>8 months old, n=183) and mink kits (<8 months old, n=184) in 2014 and 2015 (n=367). Only adult female samples were collected in the winter seasons (January - April) of 2016 and 2017 (n=82 and n=78, respectively, n=160 total). The fecal samples were collected from three pens, and so each sample may represent a total of three female mink or up to 15 mink kits. Pooled fecal samples were collected in sterile plastic bags, and were thoroughly mixed prior to RNA extraction. All fecal samples were cooled immediately after collection, and were subsequently stored at -80°C until needed.

2.2.2 RNA extraction and cDNA synthesis

Frozen fecal samples were thawed and a 67% fecal suspension was prepared (1 g feces in 0.5 mL PBS) and mixed using the Tissuelyser at 30 Hz for 4 minutes. The samples were centrifuged at 3000 x g for 15 min, and 200 µL of the supernatant was removed for RNA extraction. Viral RNA extraction was performed using TRIzol reagent according to the manufacturer's instructions (ThermoFisher Scientific). Forty µL of DEPC-treated water was added to the resulting RNA pellet and incubated at 70°C for 10 min, then mixed with a pipette to resuspend the RNA. RNA concentration was measured (NanoDrop) and was held at -80°C until needed. Reverse transcription was performed using the QuantiTect Reverse-Transcriptase cDNA synthesis kit according to manufacturer's instructions (Qiagen, Mississauga, ON, Canada). RNA samples to be tested for rotavirus were denatured at 95°C for 5 min prior to the QuantiTect reverse transcription protocol. cDNA synthesized from viral RNA was stored at -20°C until needed.

2.2.3 PCR protocols and gel imaging

PCRs were conducted using virus specific primers and annealing temperatures. Enteric mink astrovirus was detected using primers MA15 and MA17 (5'-CAAATGCCTGGAAGAACAC-3' and 5'- GAGGAGTT TCAGACAGATG-3' respectively, annealing temperature 52°C), which target a 189 bp region of the RdRp gene (Mittelholzer et al., 2003). Mink HEV was detected using primers Hep-fwd and HepE-rev (5'- CCAGAAT GGTGCTTCTATGGTGAT-3' and 5'- AATTGTTC TGCGAGCTATCAAACTC-3' respectively, annealing temperature 60°C), which target a 261 bp region of the RdRp gene (Krog et al., 2013). Rotavirus C primers RVC6-3F and C4 (5'-GTTGCATCCGTGAAGAGA ATG -3' and 5'-AGCCACATAGTTCACATTTCATCC -3' respectively, annealing temperature 59.5°C) were used to target a 356 bp region of the VP6 gene (Gouvea et al., 1991; Otto et al., 2015). The astrovirus and HEV positive controls were synthesized based on the primer targeted regions (gBlocks Gene Fragments, IDT). A confirmed rotavirus C positive porcine served as the positive control. PCR products were visualized on 2% agarose gels with 12 μL of SyberSage and a 0.1-10.0 kb DNA ladder (New England Biolabs). At least one positive rotavirus, astrovirus and HEV PCR product (if detected) from each year was sequenced to validate primer targets and to determine the variation among samples at the nucleotide level. The partial sequences of targeted genes detected in samples were compared to existing NCBI reference sequences. Cladograms were constructed for all sequenced samples, from which representative sequences were selected for further analysis based on clustering of the sequenced genes (Dereeper et al., 2008). Representative sequences from each virus detected were compared to the top 5 NCBI reference sequences (based on % identity) for all sequenced genes based on the maximum likelihood method (Dereeper et al., 2008).

2.2.4 Data analyses

Pearson Chi-Squared tests (JMP, SAS Institute) were used to statistically compare the prevalence of detected viral RNA between adult female mink and mink kits, between the two summer season collection periods (2014 and 2015, kits and adult female samples), as well as between the adult female samples collected in the summer and winter seasons. The False Discovery Rate (FDR) was applied for Chi-Squared tests comparing the prevalence of virus RNA in adult female mink samples across all years sampled (JMP Add-In). For all statistical tests conducted, a p-value ≤ 0.05 was considered significant. All phylogenetic comparisons

were conducted using Phylogeny.fr (Dereeper et al., 2008). All sequenced genes were compared, and one sequence from each cluster was used to compare to closely related viruses in a cladogram.

2.3 Results

Overall, of the 527 female mink and mink kit pooled fecal samples collected in 2014-2017, 14% (75/527) were positive for astrovirus RNA, 9% (48/527) were positive for HEV RNA, and 3% (14/527) were positive for rotavirus RNA. Twenty-four % (44/184) of pooled kit fecal samples collected in 2014 and 2015 were positive for astrovirus RNA, 18% (32/184) were positive for HEV RNA, and 2% (4/184) were positive for rotavirus RNA (**Table 2.1**). Of the 343 pooled female mink fecal samples collected in 2014-2017, 10% (34/242) were astrovirus RNA positive, 5% (18/343) were HEV RNA positive, and 4% (12/343) were rotavirus RNA positive (**Table 2.1**). Astrovirus and HEV RNA were both detected in significantly more pooled kit samples compared to pooled female samples ($p=0.01$ and $p<0.0001$, respectively) in the summer samples collected in 2014 and 2015. Considering all samples collected in 2014 and 2015, astrovirus RNA was detected in a significantly higher percentage of samples in 2014 than in 2015 ($p=0.005$). HEV RNA was detected slightly higher percentage of 2015 samples than 2014 samples ($p=0.05$). When comparing only the female samples from all years, astrovirus RNA was detected in a higher percentage of samples from the summer 2014 compared those from the winter 2016 ($q=0.02$), and from winter 2017 ($q=0.002$). When comparing all female fecal samples, astrovirus RNA was detected in a significantly higher percentage of samples collected in the summer season compared to the winter season ($p=0.001$). Samples from 2015 had a higher number of astrovirus RNA positive samples compared to samples from 2017 ($q=0.02$), and samples from 2017 had a slightly higher number of rotavirus RNA positive samples compared to those from 2016 ($q=0.05$).

In 2014, 2 adult female and 3 kit pooled fecal samples were positive for both astrovirus and HEV RNA. Two kit samples from 2015 had both astrovirus and HEV RNA, and one kit sample was positive for both rotavirus and HEV RNA. One adult female fecal sample in 2017 had detectable rotavirus and astrovirus RNA. A farm was considered positive if at least one sample was positive for any one of the screened viruses for any given year. Analysis of farm status across 50 farms showed that only one farm was rotavirus positive two

consecutive years (2016-2017), and three farms were HEV positive for two consecutive years (2014-2015, 2015-2016, and 2016-2017). No farms were positive for rotavirus or HEV for more than 2 consecutive years. In the case of astrovirus, only one farm was positive for 3 consecutive years (2014-2016), while 11 farms were positive for two consecutive years (2014-2015 only).

The cladogram constructed for 23 astrovirus partial RdRp sequences demonstrates clustering into four groups where isolate mink astrovirus (AV) 2017-ON-11az was found to be least related to other detected sequences (**Figure 2.1a**). Representative sequences (mink AV 2014-ON-7c, 2016-ON-26z, 2015-ON-8f and 2017-ON-11az) from each group were compared to mink astrovirus RdRp sequences available in GenBank (**Figure 2.1b**). Mink AV_2014-ON-7c, AV_2016-ON-26z, and AV_2015-ON-8f were most closely related to nine previously reported mink astrovirus strains, whereas mink AV_2015-ON-8f was most closely related to mink astrovirus (MiAstV) (AY179509) and mink astrovirus strain 4265 (AY196102), while also being closely related to mink astrovirus strains 4540, SMS-AstV, 4220, 4071, 4070, 3914, and 4074 (AY196104, GU985458, AY196101, AY196097, AY196096, and AY196100, respectively). Of the representative sequences, mink AV_2017-ON-11az was most distantly related to all sequenced samples and the existing GenBank sequences.

The 10 sequenced rotavirus samples clustered into two groups (**Figure 2.2a**), of which mink rotavirus (RV) 2015-ON-14g and 2015-ON-16e were used as representative sequences for further phylogenetic analysis. When compared to the VP6 gene of 17 other closely related rotavirus strains, mink RV 2015-ON-14g was most closely related to porcine rotavirus strains BRA189/04-Po (JF810442), CJ32-3 (AB889517), and CJ31-6 (AB889516), and was also closely related to porcine rotavirus C strains CUK-5 (HQ833829), CUK-6 (HQ323753), KOR/06-52-1 (KJ814477), KOR/04-105-2 (KJ814478), KOR/04-155-5 (KJ814479), KOR/09-15-7 (KJ814481), KOR/08-128-1 (KJ814483), KOR/09-15-9 (KJ814482), KOR/07-74-11 (KJ814480), 93-H5 (AB889507), 06-144-2 (FJ494691), porcine rotavirus C (GQ925781) and bovine rotavirus C strains Toyama (AB738416) and Y/3/04 (AB874633). Mink RV_2015-ON-16e was the most distantly related to all analyzed sequences (**Figure 2.2b**).

The sequences of the 13 HEV-positive samples were analyzed, and they clustered into two distinct groups (**Figure 2.3a**). Further phylogenetic analysis of representative samples mink HEV_2016-ON-38y and HEV_2017-ON-25az with 10 other closely related HEV strains showed that the representative sequences were highly related to mink HEV strains 345-3 (KC802092), 574-3 (KC802093), and 1119-3 (KC802090), and were also closely related to ferret HEV strain F63 (LC177792) (**Figure 2.3b**). Representative strains were distantly related to rat HEV strains MVZ201020 (JQ898482) and ratELOMB-131 (LC145325), wild boar HEV strains wb69-2007-gt3 (HM641319) and wb1152-08-gt3 (HM641314), and human HEV strains 1391.14 (KX757852) and 3149.14 (HM641319).

2.4 Discussion

This study aimed to assess the prevalence of astrovirus, rotavirus and HEV in Canadian commercial mink. Astrovirus and HEV RNA positive samples were found to be the most common, although positive sample numbers were modest. Overall, higher numbers of pooled fecal samples with detectable viral RNA were identified in mink kits compared to adult female mink (2014-2015), which is consistent with previous mink astrovirus and HEV research (Englund et al., 2002; Mittelholzer et al., 2003). Only astrovirus-positive samples were found to be more prevalent in the summer samples compared to the winter samples. Although viral RNA detected in the fecal samples may not represent active infections, these results indicate that the collected pooled fecal samples from kits had higher prevalence of astrovirus, rotavirus and HEV shedding. In the case of mink astrovirus, these results further demonstrate the need to monitor astrovirus shedding as a potentially important agent in pre-weaning diarrhea (Englund et al., 2002).

An increase in astrovirus shedding in the summer samples is consistent with previous studies showing an increased likelihood of enteric and other infections in the summer (Eber et al., 2011; Leekha et al., 2012; Zhang et al., 2016). The increased number of HEV infections in kits may not be directly associated with any specific clinical signs, but could contribute to the severity of disease states caused by co-infection of other bacterial or viral agents (Krog et al., 2013). Although rotavirus RNA was not highly prevalent in the tested samples, there was an increase in the prevalence of rotavirus RNA positive samples across the four years of the study (8% of total samples in 2017 compared to 0-3% of total samples in years 2014-2016), which corresponded to an apparent decrease in the number of astrovirus

RNA positive samples (1% of total samples in 2017 compared to 6-25% of total samples in years 2014-2016). For astrovirus, these patterns may not be fully representative of the extent of on-farm infection, since no kit samples were collected in the winters (2016 and 2017) and the majority of astrovirus RNA positive samples from the summer (2014 and 2015) were collected from kits. Similarly, samples from the summer season had slightly higher numbers of HEV RNA positive samples (11% of total samples) compared to samples from the winter (5% of total samples), which may also be attributed to the absence of kit fecal samples in the winter season cohort. According to farm status results, it was common for an astrovirus positive farm to remain positive for 2 consecutive years, while this event was less common for rotavirus- and HEV-positive farms. Only one farm (astrovirus RNA positive) remained positive for three consecutive years. Farms likely did not stay positive for more than two consecutive years due to the high turnover of breeding stock (i.e., most adult females are only used for two breeding cycles in Canada).

Sequences of astrovirus-positive fecal samples were highly similar between 2014 through 2016; however, the only astrovirus RdRp sequenced detected in the 2017 cohort (mink AV 2017-ON-11az) was phylogenetically distant from sequences obtained previously. The same sequence was also distantly related to nine other enteric mink astrovirus strains, with only short segments of the sequenced gene having high identity to previously reported astroviruses. The detected partial sequences of the rotavirus VP6 gene were most closely related to porcine rotavirus, which may be due to the diet of commercial mink, which is a mixed diet that may consist of pork, poultry and fish products (Krog et al., 2013; Yoon et al., 2012). Previous studies have also found a strong correlation between viruses shed in the fecal matter and the diet of animals (Fehér et al., 2014; Krog et al., 2013; Smits et al., 2013; Yoon et al., 2012). The sequenced HEV positive fecal samples in this study were highly related to three other reported mink HEV strains (345-3, 574-3 and 119-3), and also closely related to ferret HEV strain F63. This suggests that the detected HEV strains are primarily mink-specific; the risk of zoonotic transmission of mink and ferret HEV strains are currently unknown (Krog et al., 2013; Li et al., 2014).

Although the results of this study have shown low to moderate levels of astrovirus, rotavirus and HEV RNA shedding in the feces of clinically healthy Canadian commercial mink, further comparisons between healthy and diseased mink are needed to provide conclusive evidence of the role of these viruses in mink gastrointestinal health. It must be

noted that the on-farm prevalence of astrovirus, rotavirus and HEV may be higher than those reported in this study, as the pooled fecal samples used for virus RNA detection represent only a small fraction of the animals. Further, it would be interesting to correlate Aleutian disease status on-farm with shedding of these three viruses to determine whether viral co-infections outside of those discussed here would have an impact on mink health. On-farm biosecurity and personal hygiene are poor to low on most Ontario commercial mink farms (Compo et al., 2017), giving rise to concerns regarding potential cross-species transmission of viruses shed from the feces of apparently healthy mink, especially given recent reports of disease from other species (Abravanel et al., 2017; Krog et al., 2013; Martin-Latil et al., 2016; Naccache et al., 2015; Pavio et al., 2010). Virus shedding could not be related to mink coat colour because coat colour was not consistently sampled across all farms. Phylogenetic analyses of sequences showed that although most detected sequences are highly related to previously reported viral genomes, some sequences may be considered divergent and should be further investigated. While this study focused on astrovirus, rotavirus and HEV RNA shedding, a complete virome study would not only allow for further understanding of the viruses shed from healthy commercial mink, but also a better assessment of other viral agents that may be involved in mink health.

Table 2.1. Prevalence of astrovirus (AV), rotavirus (RV) and hepatitis E virus (HEV) shedding in pooled female mink and kit fecal samples collected in 2014-2017.

		Females (%)			Kits (%)		
Year	Season	AV	RV	HEV	AV	RV	HEV
2014	Summer	18 (16/87)	0	2 (2/87)	31 (27/86)	0	13 (11/86)
2015	Summer	9 (9/96)	3 (3/96)	6 (6/96)	17 (17/98)	4 (4/98)	21 (21/98)
2016	Winter*	6 (5/82)	1 (1/82)	5 (4/82)	-	-	-
2017	Winter*	1 (1/78)	8 (6/78)	5 (4/78)	-	-	-
Total (n=527):		10 (34/343)	4 (12/343)	5 (18/343)	24 (44/184)	2 (4/184)	17 (32/184)

*only pooled fecal samples from adult female mink could be collected in the winter

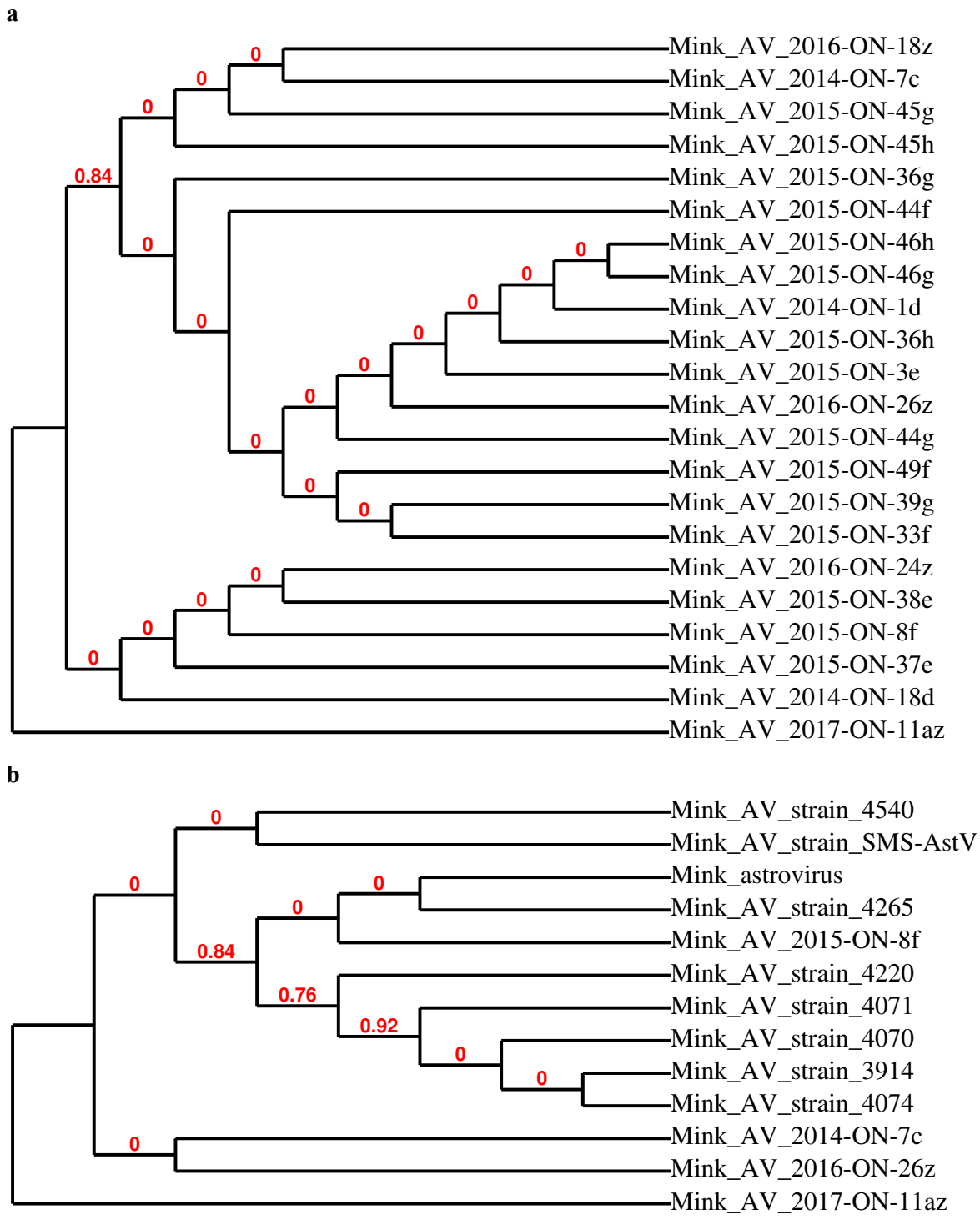


Figure 2.1. Phylogenetic relationship of mink astrovirus RdRp partial sequences (**a**) and phylogenetic analysis of mink AV 2014-ON-7c, 2015-ON-8f, 2016-ON-26z and 2017-ON-11az and closely related astrovirus strains (**b**).

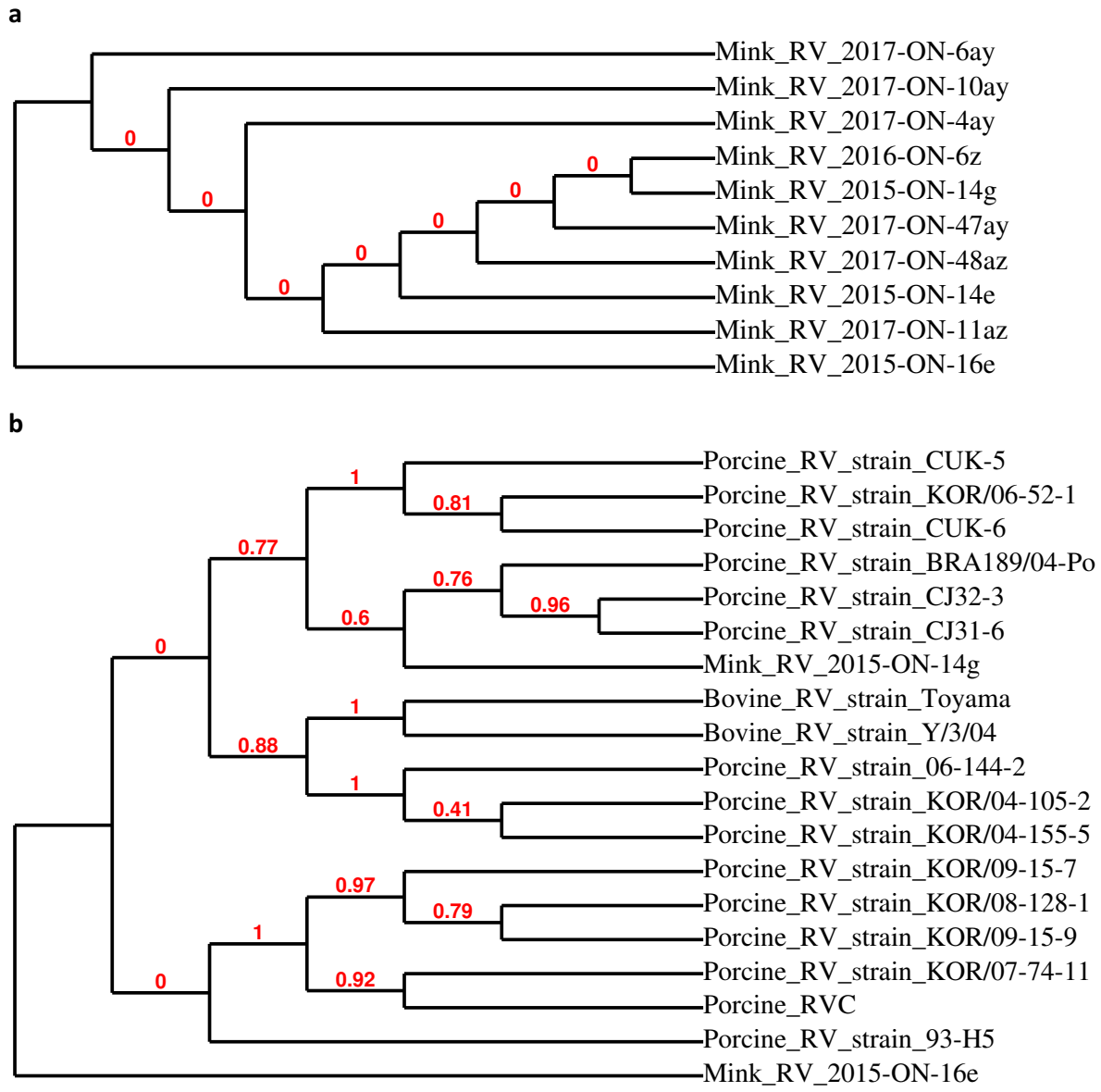


Figure 2.2. Phylogenetic relationship of detected rotavirus VP6 partial sequences (**a**) and phylogenetic analysis of mink RV 2015-ON-14g and 2015-ON-16e and closely related rotavirus strains (**b**).

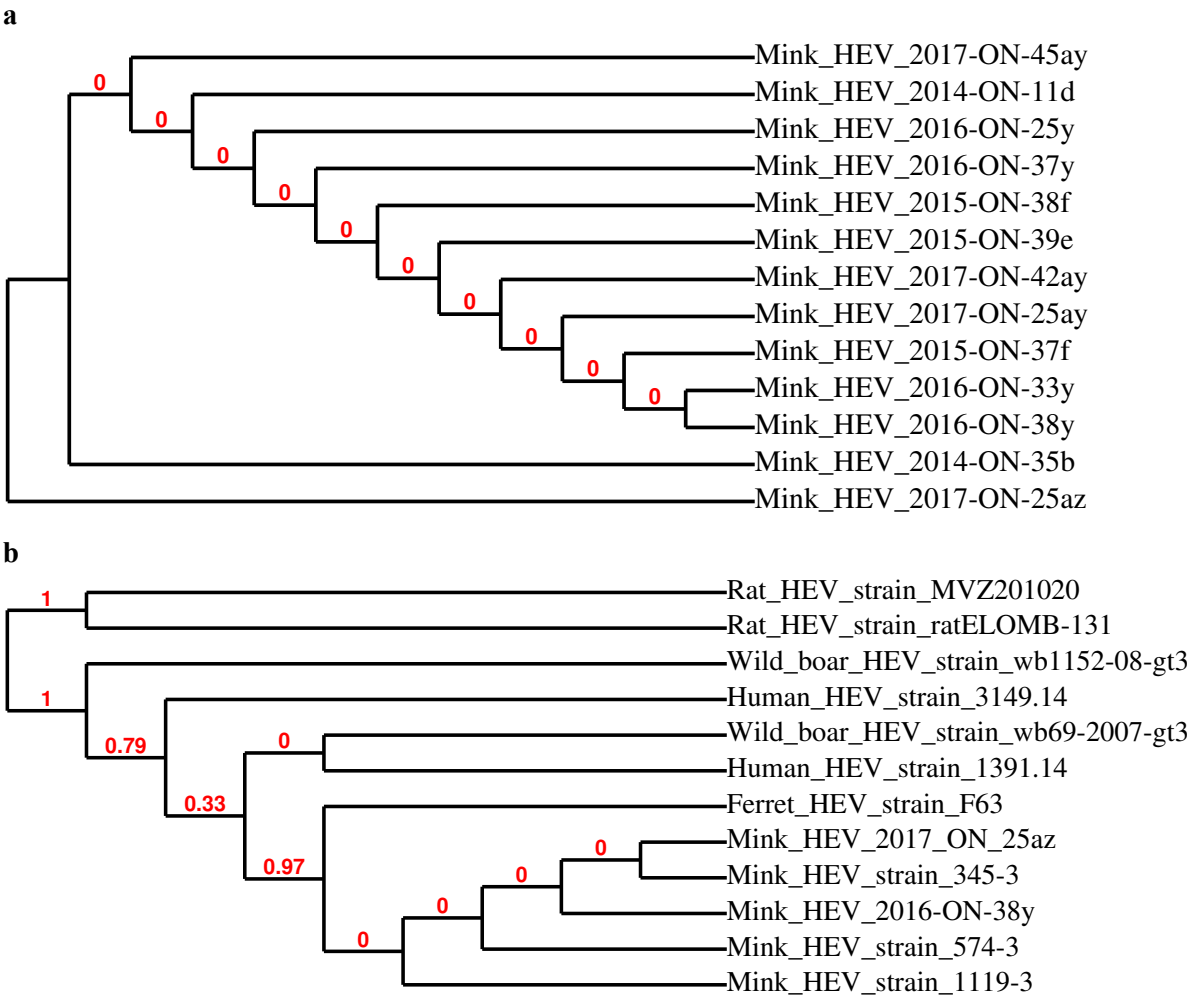


Figure 2.3. Phylogenetic relationship of mink hepatitis E virus (HEV) RdRp partial sequences (**a**) and phylogenetic analysis of mink HEV 2016-ON-38y and 2017-ON-25az and closely related HEV strains (**b**).

Chapter 3: Prevalent eukaryotic viruses and bacteriophage in Ontario farmed mink (*Neovison vison*)

Recent viral metagenomic studies have demonstrated the diversity of eukaryotic viruses and bacteriophage shed in the feces of domestic species. Although enteric disease is a major concern in the commercial mink industry, few etiologic agents have been well characterized. This study aimed to identify viruses shed in the fecal matter of clinically healthy commercial mink from 40 southern Ontario farms. Viral RNA was extracted from 67 pooled fecal samples (30 adult female mink and 37 kit) and amplified for Illumina sequencing on the NextSeq platform, and the resulting contigs were trimmed and assembled using Trimmomatic 0.36.0 and Spades 3.8.0 in iVirus (CyVerse, AZ USA) and SeqManNGen 12 (DNAStar, WI USA). Identification of assembled sequences >100 bp (Geneious 10.1.3) showed an abundance of bacteriophage sequences, mainly from families *Siphoviridae* (53%), *Podoviridae* (22%), *Myoviridae* (20%), *Inoviridae* (1%), *Leviviridae* (0.04%), *Tectiviridae* (0.01%), and *Microviridae* (0.01%). A diverse range of vertebrate viruses were detected, of which posavirus 3, mink bocavirus, gyroviruses, and avian-associated viruses were most abundant. Additionally, sequences from non-vertebrate viruses with water and soil-associated amoebal and algal hosts were also highly prevalent. The results of this study show that viruses shed in the fecal matter of healthy commercial mink are highly diverse and could be closely associated with diet, and that more research is necessary to determine how the detected viruses may impact mink health.

3.1 Introduction

In 2015, the Canadian commercial mink industry had an economic value of $CAD 98 million, with approximately 3 million mink pelts produced (Statistics Canada. Table 003-0015, 2016; Statistics Canada. Table 003-0014, 2016). Of 213 total Canadian mink farms, 46 farms are located in Ontario (Statistics Canada. Table 003-0015, 2016). Enteritis in mink is a generalized condition thought to be caused by multiple viral and bacterial agents, and outbreaks of diarrhea on farms pose significant economic risk to producers (Englund et al., 2002; Gorham et al., 1990). Viral infections have been shown to play an important role in mink enteritis (Xie et al., 2017), although few have been well characterized. Viral agents known to cause enteric disease in mink include astrovirus, rotavirus and mink enteritis virus

(MEV), (Arnold et al., 1997; Englund et al., 2002; Otto et al., 2015; Reynolds, 1969; Wang et al., 2015) although the prevalence of these viruses has not been monitored in Canada.

Recent viral metagenomic (virome) studies have revealed that domestic and wild animals harbor a wide variety of divergent and novel viral species and strains, as well as viruses previously characterized and associated with disease (Bodewes et al., 2014; Duarte et al., 2013; Fehér et al., 2014; Martella et al., 2011; Ng et al., 2014; Shan et al., 2011; Zhang et al., 2014). These studies have highlighted the similarity of viromes between species with comparable diets (carnivores, omnivores), and the high prevalence of zoonotic viruses, such as hepatitis E virus (rabbits, swine) and human gyroviruses (ferret) (Fehér et al., 2014; Kasorndorkbua et al., 2004; Lhomme et al., 2013). In addition to mammalian viruses, many viral metagenomic studies have also reported a high prevalence of insect-associated viruses and bacteriophages (Colomer-Lluch et al., 2011b; Fancello et al., 2014; Rolain et al., 2011). Recently, bacteriophages have been gaining attention for their potential to transduce and spread antimicrobial-resistance genes (ARGs) to different bacterial populations, and there is a strong correlation between antimicrobial use and an increased number of phage-encoded ARGs (Allen et al., 2011; Modi et al., 2014). Overuse of antimicrobials in the commercial industry has resulted in a widespread increase of antimicrobial resistant (AMR) bacterial populations, but the role of phage as potential carriers of resistance genes is still not well understood (Allen et al., 2011; Enault et al., 2017; Modi et al., 2014). Although the transduction of functional ARGs from phage to bacteria may be a rare event (Enault et al., 2017), it would be valuable to monitor and correlate the use of antimicrobials and the presence of phage-encoded ARGs on farms.

As mink mortality is a production concern, identifying viruses that may play a role in mink health and disease would further the understanding of agents involved in mink enteritis. Additionally, assessment of prevalent bacteriophages may provide insight into the bacterial populations that can cause disease in mink, and help to understand the relationship between phage and bacterial populations. The first objective of this study is to identify the prevalent mammalian, environmental and phage viruses shed in the feces from clinically healthy commercial adult female mink and mink kits from 40 Ontario farms. The second objective is to identify phage-encoded antimicrobial resistance genes from the detected phage sequences, and compare those identified to antimicrobial resistance data from bacterial isolates from subsequent years sampled.

3.2 Materials and methods

3.2.1 Sample collection, dilution and filtration

Sixty-seven pooled fecal samples were collected between July and October of 2014 from 40 Ontario mink farms. Thirty-seven pooled kit fecal samples and 30 pooled adult female fecal samples were collected from under three pens, representing up to three adult female mink per sample or up to 15 mink kits per sample. Information on farm location, recent history of antimicrobial use, and mink coat colour was collected for each farm. Samples were collected in plastic bags and stored at -80°C until processing. To prepare the 10% fecal sample dilution, the samples were thawed and mixed thoroughly in the bag, then 1 g of fecal matter was added to 9 ml of PBS. The sample was then centrifuged at 10,000 x *g* for 15 min at 4°C to remove large particulates and bacteria. The supernatant was removed, filtered (Millipore syringe 0.45 μm filters) and stored at -20°C.

3.2.2 Purification and extraction of viral nucleic acids

To reduce non-viral nucleic acids, 200 μL of filtered supernatant was treated with a nuclease mixture of 7 μL TURBO DNase (Ambion, Life Technologies, Grand Island, NY, USA), 3 μL Baseline-ZERO (Epicentre, Chicago, IL, USA), and 1 μL of diluted RNase T1 (Fermentas Canada Inc., Burlington, ON) in 7 μL 1× DNase buffer (Ambion, Life Technologies, Grand Island, NY, USA). This mixture was incubated at 37°C for 90 min (Victoria et al., 2009; Zhang et al., 2014). DNase and Baseline-Zero were inactivated by incubating for 20 min at 70°C. RNase T1 was inactivated by a lysate in the first step of nucleic acid extraction. Viral nucleic acids were extracted from 200 μL of the DNase and RNase treated product (Invitrogen Viral RNA/DNA Extraction Kit). In the purification procedure, 20 μL of RNase-free water was used to elute nucleic acids.

3.2.3 Viral cDNA synthesis and pre-amplification enrichment of viral cDNA and DNA

Ten μL of extracted viral nucleic acids was incubated with 100 pmol of a primer consisting of a fixed 18 bp sequence with a random nonamer at the 3' end (GCCGACTAATGCGTAGTCNNNNNNNN) for 2 min at 85°C. cDNA synthesis was performed using reverse transcriptase from the Quantitect Reverse Transcription kit according to manufacturer's instructions. For pre-PCR amplification enrichment of viral

cDNA and DNA,10 µL of the cDNA synthesis product was incubated with 50 pmol of the previously described random primer at 92°C for 2 min, 4°C for 2 min, then with 5U of Klenow fragment with 1x Klenow Buffer (New England Biolabs, Ipswich, MA, USA) at 37°C for 1 h (Li et al., 2015).

3.2.4 PCR amplification and product purification

Klenow products were PCR amplified using KAPA 2G HotStart ReadyMix (KAPABiosystems, Boston, MA, USA). Five µL of the Klenow product was mixed with 1 µL of 2.5 mM a primer containing only the 18 bp fixed portion (GCCGACTAATGCGTAGT C) of the previously described primer. An additional 1 µL of 25 mM of $MgCl_2$ was added to the KAPA master mix. Temperature cycling was performed as follows: 1 cycle of 95°C for 5 min, 33 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 90 s. Samples were kept at 72°C for an additional 10 min of extension and held at 4°C at the end of the run. PCR products were purified once using the Agencourt AMPure XP beads (Beckman Coulter, Brea, CA, United States) with a 0.8:1 ratio of beads to sample. Eighty % ethanol was used for the ethanol wash and 32 µL of elution buffer was used to extract purified DNA fragments from beads.

3.2.5 NGS library preparation and sequence data analyses

Sixty-seven samples (kit n = 37, adult female n = 30) were prepared for NGS using Nextera XT DNA Sample Preparation Kit (Illumina, San Diego, CA, USA). Samples were sequenced using Illumina NextSeq500 V2 chemistry on a 2x125 cycle (Donnelly Centre, Toronto ON), and reads were demultiplexed by vendor software (Donnelly Centre, Toronto ON). Low quality reads were filtered using Trimmomatic 0.36.0 in iVirus (CyVerse, AZ USA) using default parameters. Trimmomatic output was used for *de novo* assembly in Spades 3.8.0 (CyVerse, AZ USA) using Kmer size 65, and SeqManNGen 12 (DNAStar, Madison, WI USA) (Zhang et al., 2014). Assembled contigs greater than 700 bp were aligned to the NCBI viral reference database (viral1.1.genomic.fna.gz) using BLASTn in Geneious 10.1.3 with an *E* value cut-off 10^{-4}. The resulting reads that aligned over at least 100 bp with a reference viral sequence were compiled and used for further analysis. Top phage and non-phage viral families were identified for all sample libraries, and the sequences from specific viruses which had the highest prevalences were compared between for adult females and kits, and between farms grouped based on 5 geographical regions (JMP, SAS Institute) (**Appendix**

B). The Comprehensive Antibiotic Resistance Database (CARD) was used to identify ARGs in all phage sequences, using an E-value cutoff of $1e^{-5}$. Only sequences with amino acid identity of ≥80%, query coverage ≥85%, bit-score ≥70, and reference sequence coverage of ≥40% were considered for further analysis (Enault et al., 2017; Gibson et al., 2015). The most prevalent vertebrate virus sequences were further assessed based on identity, sequence length and prevalence across samples. Viral sequences with lower levels of similarity in amino acid identity (average identity <90%) were then compared to these reference viral sequences (GenBank) to identify the level of identity of protein-encoding genes. All detected sequences for each virus with low average identity were used for *de novo* assembly in Geneious 10.1.3, followed by phylogenetic analysis in phylogeny.fr with their closest related viral sequences (BLASTn hits with the highest identity) (Dereeper et al., 2008).

3.2.6 Antimicrobial testing of bacterial isolates

A total of 154 pooled fecal samples collected in 2016 (n=76) and 2017 (n=78) were used for AMR testing of *E. coli* and *Salmonella* bacterial isolates. The pooled fecal sample was thoroughly mixed in the collection bag, then 1 g was aliquoted into a sterile bag for AMR testing at Public Health Agency of Canada (PHAC) using the culturing and testing methods described in the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS, 2014). Outcomes of AMR testing were evaluated using upper (UL) and lower (LL) Sterne limits. Only isolates classified as intermediate resistance (I) or resistant (R) were used for further comparisons.

3.2.7 Statistical analysis

JMP (SAS Institute) was used to conduct one-way non-parametric Wilcoxon tests to compare the relative abundances of top phage and mammalian viral sequences between adult female mink and mink kits. For all statistical tests conducted, a p-value ≤0.05 is considered significant. Information collected on mink coat colour was not used for statistical analysis due to inconsistent sampling.

3.3 Results

3.3.1 Prevalent phage sequences

A total of 345,127 assembled reads (>700 bp) were extracted for comparison to GenBank's viral database. Of the reads with detectable similarity to existing viral reference genomes, those ≥100 bp (112,144) were used for further analysis. Ninety-eight % of reads ≥100 bp aligned to bacteriophage sequences (109,612 sequences). Phage sequences were compiled and analyzed based on bacterial host. The most prevalent sequences were identified to have *Bacillus, Clostridium, Enterococcus*, *Escherichia*, *Lactobacillus, Lactococcus*, *Proteus, Pseudomonas, Salmonella*, *Shigella*, *Staphylococcus*, and *Streptococcus* bacterial hosts, with the top three bacteriophage species from each group listed in **Table 3.1**. *Escherichia* and *Enterococcus*-associated bacteriophage sequences had the highest identities to GenBank reference sequences (84-94%).

Seven viral families were identified in 12 most prevalent bacteriophage groups (76,558 sequences), including *Siphoviridae* (53%), *Podoviridae* (22%), *Myoviridae* (20%), *Inoviridae* (1%), *Leviviridae* (0.04%), *Tectiviridae* (0.01%), and *Microviridae* (0.01%). An additional 4.8% of detected bacteriophage sequences were unclassified, with the majority belonging to the order *Caudovirales*. *Pseudomonas* phage sequences were found to be significantly higher in adult female mink samples ($p = 0.02$), and no other significant differences were found in other detected phage sequences between age groups.

3.3.2 Phage encoded antimicrobial-resistance genes and antimicrobial resistance results

Phage sequences were also screened for antimicrobial resistance genes using the CARD database. A total of 611 ARG sequences were detected in phage sequences using an E-value cutoff of $1e^{-5}$. Applying conservative search parameters resulted in 42 ARG sequences associated with 17 unique resistance gene mutations (**Table 3.2**). *Salmonella enterica* (*S. enterica*) rrsD gene 16S rRNA mutations and *Escherichia* species-associated ARGs were the most common (15 each), followed by *Neisseria* (5), *Mycobacterium* (4), *Clostridium* (2) and *Pseudomonas* (1) species-associated ARGs. The detected ARGs were most commonly associated with resistance to spectinomycin (**Table 3.2**).

E. coli was successfully isolated from 22 samples of the 154 pooled 2016 and 2017 fecal samples for AMR testing (12 from 2016 and 10 from 2017), no *Salmonella* isolates were obtained. Seven *E. coli* isolates were found to have intermediate resistance or were

resistant to at least one of the tested antimicrobials (3 from 2016 and 4 from 2014), with 6 isolates found to be resistant to tetracycline. The remaining 15 isolates were not found to be resistant to any of the tested antimicrobials. When comparing 2016 and 2017 AMR results and the detected ARGs from the 2014 pooled fecal samples, 3 *E. coli* isolates were resistant to spectinomycin, and two of the same isolates were found to be resistant to gentamicin, for which *E. coli*-associated ARGs were also detected in phage sequences (**Table 3.3**).

3.3.3 Prevalent non-phage viral sequences

Of 2,532 non-phage sequences, 49% (1,237) aligned to vertebrate viruses. The most prevalent of the vertebrate viruses were from viral families *Parvoviridae*, *Circoviridae*, *Genomoviridae*, and *Herpesviridae*. Vertebrate viral sequences detected with the highest identity (>92%) to previously reported viruses include posavirus 3 (KR019688), mink bocavirus (KU950356), chicken anemia virus (HM590588), avian gyrovirus 2 (JQ690763), avian adeno-associated virus strains DA-1 and ATCC VR-865 (AY629583 and AY186198, respectively), gyrovirus 4 (KY024580), gyrovirus GyV3 (JQ308210) (**Table 3.4**). Sequences with relatively low identity to saimiriine herpesvirus 2 (AH003100.2), chimpanzee faeces-associated virus 1 (KR704911), *Gemykibivirus* HCBI8.215 virus (LK931483), *Desmodus rotundus* parvovirus (KX907333), gyrovirus Tu243 (KF294861), chicken parvovirus ABU-P1 (GU214704) and chicken associated smacovirus (KY086299) were also detected in high numbers (**Table 3.5**). No significant differences were found when considering vertebrate viral sequence prevalence between female mink and kit samples or between farm groups.

In addition to vertebrate viruses, 51% (1,295/2,532) of non-phage sequences were associated with non-vertebrate hosts, including environment-associated viruses (water, algae and soil), and plant, insect, fungal or crustacean-associated hosts. The 10 most prevalent of the non-vertebrate viral sequences include algae, amoeba, insect and crustacean associated viruses, with 18-63% prevalence in samples (**Table 3.6**). Megavirus courdo11 (JX975216), and Tokyovirus A1 (AP017398) are water-associated amoebal viruses, while mimivirus terra2 (KF527228) is a soil-associated ameobal virus. *Cafeteria roenbergensis* virus BV-PW1 (GU244497), *Aureococcus anophagefferens* virus isolate BtV-01 (KJ645900), *Chrysochromulina ericina* virus isolate CeV-01B (KT820662), and *Acanthocystis turfacea* Chlorella virus 1 (AY971002) are water-associated viruses with algae as hosts. White spot syndrome virus strain CN01 (KT995472) affects shrimp, while *Culex pipiens* densovirus

(FJ810126) and *Melanoplus sanguinipes* entomopoxvirus (AF063866) are associated with mosquitos and grasshoppers, respectively.

3.3.4 Analysis of vertebrate viral sequences with low average identity

This study identified sequences from 7 prevalent viruses that had low average identity (<90%) to the reference sequences of vertebrate viruses. The average identities of detected sequences, their prevalence in samples, as well as query-encoded proteins are listed in **Table 3.5**. **Figure 3.1a** shows the phylogenetic relationship between the consensus sequence (Herpesvirus 2014-ON_consensus) from *de novo* assembly of all detected sequences with similarity to saimiriine herpesvirus 2 and viruses with similar genome structure. Herpesvirus 2014-ON_consensus was most closely related saimiriine herpesvirus 2 and also clustered closely with ateline herpesvirus 3 (AF083424), but was distinct from varicella-zoster virus (AH002362.2) (Albrecht et al., 1992). Analysis of individual viral sequences with highest similarity to an unclassified virus, chimpanzee faeces-associated virus 1 CPNG_29286 (KR704911), showed segments with 100% and 76% identity to genes encoding replication-associated proteins in chimpanzee faeces-associated circular DNA virus 1 CPNG_29286 and chimpanzee faeces-associated circular DNA virus 1 CPNG_29268 (KR704711). **Figure 3.1b** shows that the assembled consensus sequence (faeces-associated circular virus 2-14-ON_consensus) clusters closely with 8 other strains of chimpanzee stool-associated circular viruses (GQ351272-8, KR704912), but was distinct from human stool-associated circular virus NG13 (GQ404856). **Figure 3.1c** shows that the assembled consensus sequence (HCBI8.215-like virus 2014-ON_consensus) are more closely related to torque teno virus strain TTV-HD14a (FR751463) compared to HCBI8.215 virus (LK931483) and HCBI9.212 virus (LK931484). Sequences with highest similarity to *Desmodus rotundus* parvovirus strain DRA25 (KX907333, *Parvoviridae*) were used in *de novo* assembly, resulting in chapparvovirus 2014-ON_consensus. This consensus sequence was not closely related to the *Desmodus rotundus* parvovirus strain DRA25 genome or three other parvoviruses with similar structure (KF925531, KX272741, and JX885610) (**Figure 3.1d**) (Souza et al., 2017). Sequences with highest similarity to gyrovirus Tu243 (KF294861, *Circoviridae*) had segments with 100% identity to gyrovirus Tu243 VP1 and VP2 genes, as well as 63% identity to the VP1 gene of gyrovirus 4. The longest sequence had 64% identity over 76% (1530/2020) of the gyrovirus Tu243 genome. Phylogenetic analysis shows that assembled sequence (gyrovirus 2014-ON_consensus) clustered most closely with gyrovirus Tu243 and

gyrovirus 4 strain D137 (JX310702), and was also closely related to gyrovirus GyV3 (JQ308210), avian gyrovirus 2 (HM590588), human gyrovirus 1 strain 915 F 06 007 FD (FR823283), gyrovirus Tu789 (KF294862) and chicken anemia virus (M55918) (**Figure 3.1e**) (Chu et al., 2013; Phan et al., 2014; Sauvage et al., 2011). Segments of detected sequences had 100% identity to NS1, VP1 and VP2 proteins. The assembled sequences with highest similarity to chicken parvovirus ABU-P1 (GU214704, *Parvoviridae*) (parvovirus 2014-ON_consensus) was highly related to the genomes of chicken parvovirus ABU-P1 and turkey parvovirus 260 (GU214706), which clustered separately from turkey parvovirus 1078 (GU214705) (**Figure 3.1f**) (Day and Zsak, 2010). Sequences with highest similarity to chicken-associated smacovirus strain RS/BR/2015/4 (KY086299, unclassified) encoded for capsid and replication-associated proteins with 100% and 95% identities to chicken-associated smacovirus strain RS/BR/2015/4 and human smacovirus (AJF23075). In phylogenetic analysis, smacovirus 2014-ON_consensus sequence clustered most closely with chicken-associated smacovirus strains RS/BR/2015/1- RS/BR/2015/4 (KY086298-KY086301), but were also closely related to bovine faeces-associated smacovirus strain GP3_46075_cow (KT86222) and human smacovirus 1 isolate Virginia/2/2012/Chesapeake/J23 (KP233186) (**Figure 3.1g**).

3.4 Discussion

This study is a preliminary assessment of viral sequences in the fecal matter of healthy commercial mink on 40 Ontario farms, and the diversity of bacteriophage and eukaryotic virus sequences was fairly consistent with previous research on the fecal virome of carnivorous species (Fehér et al., 2014; Ng et al., 2014; Phan et al., 2015; Smits et al., 2013; Zhang et al., 2014). The 12 most prevalent phage viruses detected in this study represent 70% (76,558/109612) of all detected phage sequences. Compo et al. (2017) conducted a study on the fecal microbiome of Ontario commercial mink sampled in 2014, and comparison between the abundance of the phage sequences and their respective bacterial hosts show that *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Clostridium*, *Escherichia*, *Streptococcus* and *Pseudomonas* species were also prevalent in the mink microbiome. *Bacillus*, *Salmonella*, *Shigella*, *Staphylococcus*, and *Proteus* bacterial populations were not found to be highly prevalent in the mink fecal microbiome study (Compo et al. 2017). Interestingly, significantly higher numbers of *Pseudomonas*-associated phage sequences were detected in adult female fecal samples compared to kit samples ($q = 0.02$), but since the

number of detected sequences does not represent infections by *Pseudomonas* species, the implications of these results are unclear. Although previous studies have shown that lytic phage therapies may be useful in controlling *Pseudomonas* bacterial populations (Cao et al., 2015; Gu et al., 2016), further investigation is required to understand the natural role that the associated bacteriophage species play in bacterial populations. Due to increasing number of antimicrobial resistant bacterial strains, bacteriophage therapy may prove to be an effective alternative to target bacterial species that can cause disease in mink (Cao et al., 2015).

Based on previous evidence that phage encoded ARGs are rare (Enault et al., 2017), conservative filtering parameters were used to sort ARG sequences in phage sequences. The detected ARGs were manually searched against viral and phage databases in NCBI, and it was found that many of identified in phage sequences have never been previously identified in phage sequences (PmrC, EF-Tu and rRNA mutations). Since phage would gain no apparent evolutionary advantage in integrating these ARGs as phage are not directly affected by antimicrobials, it is unlikely that the phage in our samples carried fully functional resistance genes (Enault et al., 2017). It is more likely that the ARG sequences that have been previously identified in phage or prophage (acrD and mdtB) represent actual integration events; however, it is not likely that these ARGs can be transduced to bacteria and function as resistance genes, since functional ARGs in viruses are often only the result of long-term high-dose use of antimicrobials (Enault et al., 2017; Modi et al., 2014). These ARGs may be the result of specialized transducing phage or randomized integration events. The use of antimicrobials has been previously shown to increase the number of phage- and prophage-encoded ARGs, which increases the likelihood of horizontal transfer of functional resistance genes to bacterial populations (Allen et al., 2011; Enault et al., 2017; Schwarz, 1994). AMR testing of pooled fecal samples from 2014 was not available; however, comparisons were drawn between the results of the AMR testing results from fecal samples collected in 2016-2017 to the identified phage-encoded ARGs from the fecal samples collected in 2014. Gentamicin and streptomycin resistance was identified in the bacterial isolates, and the corresponding resistance genes were identified in phage sequences. Although previous evidence indicates that it is rare for phage to integrate resistance genes from bacterial hosts (Enault et al., 2017), these results suggests that antimicrobial resistance in the bacterial population may still be related to the transduction of resistance genes in phage. Producers were asked to report the use of antimicrobials on farms, but due to only partial completion of the survey, the information collected on antimicrobial use from the 2014 sample cohort may

not be fully representative. One farm was documented to have treated animals with in-feed penicillin, but beta-lactamase genes were not among the 17 ARG sequences detected in the phage sequences.

This study found the highest number of viral sequences from the families *Herpesviridae*, *Parvoviridae*, *Circoviridae*, *Anelloviridae* and *Picornaviridae*. Previous fecal virome studies in ferrets and felids have also found high numbers of viral sequences belonging to the families *Parvoviridae*, *Anelloviridae* and *Picornaviridae*, but have also detected sequences from the families *Astroviridae*, *Reoviridae*, *Hepeviridae*, *Papillomaviridae*, *Picobirnaviridae*, and *Coronaviridae* (Fehér et al., 2014; Ng et al., 2014; Smits et al., 2013; Zhang et al., 2014). High numbers of sequences with 84-96% identity to posavirus 3 strain 958-4 were identified, which has been previously detected in fecal samples collected from commercial swine in high-density farms (Hause et al., 2015). Hause et al. (2015) suggest that this strain of posavirus is derived from nematodes parasitizing commercial swine. The detected posavirus sequences may be the result of contamination from the soil at the time of fecal sample collection, but also could be attributed to the mink diet, which often consists of pork and poultry products, or nematode infections in the mink gut (Krog et al., 2013). Similarly, the identified avian-associated viral sequences (chicken anemia virus, parvovirus, smacovirus, and avian adeno-associated virus) were most likely the result of mink diet. Previous evidence from viral metagenomic studies and case reports in ferrets, felids, mink and other wild carnivores have also correlated the presence of avian viruses and swine viruses in fecal samples to the diet of the animals (Bodewes et al., 2014; Fehér et al., 2014; Krog et al., 2013; Smits et al., 2013). This is also the first report of mink bocavirus sequences in commercial mink fecal samples in Canada, with 98-100% identity to the strain identified in 2016 in China (Yang et al., 2016). This strain was most closely related to feline bocavirus (JQ692585). Yang et al. (2016) found no correlation between mink bocavirus and diarrhea, but stated that these results may not be fully representative due to the small sample size.

Viruses with low average identity were used in *de novo* assembly and the resulting consensus sequences were compared to closely related viruses. Most consensus sequences found to be closely related to the initial best BLASTn hit of the individual sequences, with the exception of HCBI8.215-like virus 2014-ON_consensus and chapparvovirus 2014-ON_consensus. HCBI8.215-like virus 2014-ON_consensus was found to be more closely

related to torque teno virus strain TTV-HD14a. Chapparvovirus 2014-ON_consensus did not cluster with the initial best BLASTn hit, *Desmodus rotundus* parvovirus strain DRA25, or three other parvoviruses with similar not closely related to *Eidolon helvum* parvovirus 2 isolate Parvo_th_node176_9_9_893755, rat parvovirus 2 strain 9 or turkey parvovirus TP1-2012/HUN, indicating that it could be a novel mink parvovirus. Aside from mink bocavirus, the other prevalent vertebrate viruses identified in this study have been previously isolated in other species. HCBI8.215 virus was first isolated from the serum of healthy cattle, and gyrovirus Tu243 and GyV3 were isolated from human fecal samples (Lamberto et al., 2014; Phan et al., 2014; Tung G. Phan et al., 2012). Six of the 15 prevalent vertebrate viruses described in this study are of avian origin. Although virus shedding does not represent active infections, some of the viruses identified in this study may have the potential to be transmitted to the humans, commercial and wild animals in close proximity to mink farms due to poor biosecurity (Compo et al., 2017).

In conclusion, this viral metagenomic study provides a preliminary overview of the commercial mink fecal virome, showing a diverse range of bacteriophage and eukaryotic virus sequences, including a potentially novel chapparvovirus. It is not known whether the detected bacteriophage and eukaryotic virus sequences represent commensal species, or if these viruses are capable of influencing bacterial populations and causing disease in mink. Further research is required to clarify the phylogeny of low-identity sequences identified in this study and to determine the role of these prevalent viruses in mink health.

Table 3.1. Twelve most prevalent bacteriophage sequences based on bacterial host.

Phage group	% of total phage sequences (109612)	Species detected	Top 3 most prevalent species	Average identity (%)	Accession
Escherichia phage	16	228	*Enterobacteria* phage phiEcoM-GJ1	85	EF460875
			Enterobacteria phage RTP	84	AM156909
			Enterobacteria phage vB_EcoS_NBD2	72	KX130668
Enterococcus phage	11	44	*Enterococcus* phage EFDG1	87	KP339049
			Enterococcus phage IME_EF3	84	KF728385.2
			Enterococcus phage VD13	86	KJ094032.2
Bacillus phage	7	94	*Bacillus* phage B103	69	X99260
			Bacillus phage BCJA1c	71	AY616446
			Bacillus phage vB_BhaS-171	69	KU160496
Staphylococcus phage	6	121	*Staphylococcus* phage 6ec	72	KJ804259
			Staphylococcus phage vB_SepS_SEP9	72	KF929199
			Staphylococcus phage Stau2	71	KP881332
Lactococcus phage	6	83	*Lactococcus* phage Tuc2009	71	AF109874.2
			Lactococcus phage 1706	69	EU081845
			Lactococcus phage GE1	72	KT339177
Pseudomonas phage	4	105	*Pseudomonas* phage Pf3	70	M11912
			Pseudomonas phage vB_PsyM_KIL1	73	KU130126
			Pseudomonas phage JBD44	71	KU199710
Streptococcus phage	4	86	*Streptococcus* phage phiARI0923	70	KT337370
			Streptococcus phage SpSL1	72	KM882824
			Streptococcus virus 9872	71	KU678390
Salmonella phage	4	80	*Salmonella* phage 9NA	69	KJ802832
			Salmonella phage 64795_sal3	86	KX017520
			Salmonella phage IME207	84	KX523699.2
Clostridium phage	3	51	*Clostridium* phage 39-O	89	EU588980
			Clostridium phage c-st	74	EU588980
			Clostridium phage phiCT19406C	72	KM983332
Lactobacillus phage	3	45	*Lactobacillus* phage phiJL-1	72	AY236756
			Lactobacillus phage AQ113	69	HE956704
			Lactobacillus phage PLE3	76	KU848186
Proteus phage	2	9	*Proteus* phage PM 75	88	KM819694
			Proteus phage PM16	64	KF319020
			Proteus phage vB_PmiM_Pm5461	75	KP890823
Shigella phage	2	21	*Shigella* phage pSf-1	84	KC710998
			Shigella phage pSf-2	88	KP085586
			Shigella phage SP18	94	GQ981382

Table 3.2. Antimicrobial resistance genes (ARGs) identified in phage sequences using conservative filtering parameters (Enault et al. 2017).

Detected ARG mutations	Associated antimicrobial	Sequences detected	Coverage of reference gene (%)	Average identity (%)	Average Bit-Score
Clostridium difficile EF-Tu	Elfamycin	2	68	80	742
Escherichia coli str. K-12 rrsB gene 16s rRNA	Gentamicin	3	52	84	819
Escherichia coli str. K-12 rrsC gene 16s rRNA	Kasugamycin	2	42	86	728
Escherichia coli str. K-12 rrsH gene 16s rRNA	Spectinomycin	2	56	95	1365
Escherichia coli str. K-12 MC4100 glpT	Fosfomycin	1	62	85	941
Escherichia coli str. K-12 MG1655 mdtB	Multi-drug	1	99	80	939
Escherichia coli str. K-12 MG1655 yojI	Microcin	2	52	84	905
Escherichia coli str. K-12 W3110 acrD	Multi-drug	1	63	81	618
Escherichia coli str. K-12 W3110 PmrC	Polymycin	1	53	92	1258
Escherichia coli str. K-12 CFT073 EF-Tu	Pulvomycin	1	68	94	1283
Escherichia coli str. K-12 MG1655 EF-Tu	Enacyloxin	1	65	94	1188
Mycobacterium abscessus 16S rRNA	Kanamycin	1	60	80	798
Mycobacterium chelonae 16S rRNA	Neomycin	2	61	82	827
Mycobacterium tuberculosis H37Rv 16S rRNA	Viomycin	1	47	82	692
Neisseria meningitidis 16S rRNA	Spectinomycin	5	52	85	890
Pseudomonas aeruginosa gyrA and parC	Fluoroquinolone	1	47	82	1051
Salmonella enterica rrsD gene 16S rRNA	Spectinomycin	15	67	93	1589

Table 3.3. Antimicrobial resistance testing of *E. coli* isolates from pooled fecal samples collected in 2016 and 2017, where dashes represent susceptible isolates, I represents isolates with intermediate resistance, and R represents resistant isolates.

	Isolate ID						
	160008	160028	160055	170015	170055	170056	170059
Amoxicillin/ Clavulanic Acid	-	-	-	-	-	>32, R	-
Ampicillin	-	-	-	-	>32, R	>32, R	-
Cefoxitin	-	-	-	-	-	>32, R	-
Ceftriaxone	-	-	-	-	-	2, I	-
Ciprofloxacin	-	0.12, I	-	-	-	-	-
Gentamicin	-	-	-	-	>16, R	>16, R	-
Streptomycin	-	64, R	-	-	64, R	64, R	-
Sulfisoxazole	>256, R	-	-	-		>256, R	>256, R
Tetracycline	>32, R	-	>32, R	>32, R	>32, R	>32, R	>32, R
Trimethoprim/ Sulphamethoxazole	>4, R	-	-	-	-	-	>4, R

Table 3.4. Detected vertebrate viruses with the highest identity to previously reported viruses and their prevalence in samples.

Detected virus	Accession number	% of total vertebrate viral sequences (1237)	Average identity % (range)	Prevalence in samples (%, n=67)
Posavirus 3 strain 958-4	KR019688	11	93 (84-96)	7
Mink bocavirus	KU950356	11	98 (74-100)	49
Chicken anemia virus	HM590588	7	97 (73-99)	63
Avian gyrovirus 2	JQ69076	4	97 (91-100)	54
Avian adeno-associated virus strain DA-1	AY629583	3	92 (70-98)	43
Avian adeno-associated virus ATCC VR-865	AY186198	2	92 (77-97)	28
Gyrovirus 4 strain RS/BR/15	KY024580	0.3	96 (89-100)	6
Gyrovirus GyV3	JQ308210	0.3	94 (81-99)	6

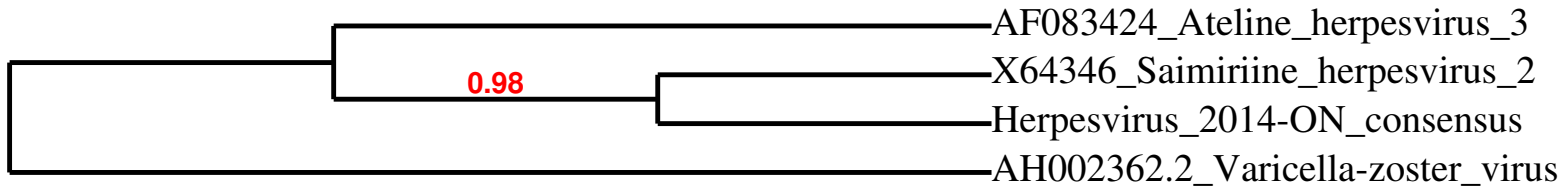
Table 3.5. Detected vertebrate viral sequences with low identity to previously reported viruses, their prevalence in 67 samples, and the protein encoding genes detected in the query sequences.

Detected virus	Accession number	% of total vertebrate viral sequences (1237)	Prevalence in samples (%)	Average identity (%)	Sequence encoded proteins (% identity)
Saimiriine herpesvirus 2	AH003100.2	7	52	71	Thymidylate synthase (100%)
Chimpanzee faeces-associated virus 1 CPNG_29286	KR704911	5	43	70	Replication-associated proteins (100%)
HCBI8.215 virus	LK931483	4	25	89	Capsid and replication-associated proteins (100%)
D. rotundus parvovirus strain DRA25	KX907333	3	39	68	NS1 and capsid protein 1 (100%)
Gyrovius Tu243	KF294861	3	39	67	VP1 and VP2 (100%)
Chicken parvovirus ABU-P1	GU214704	2	24	71	NS1, VP1 and VP2 (100%)
Chicken associated smacovirus strain RS/BR/2015/4	KY086299	2	22	89	Capsid and replication-associated proteins (100%)

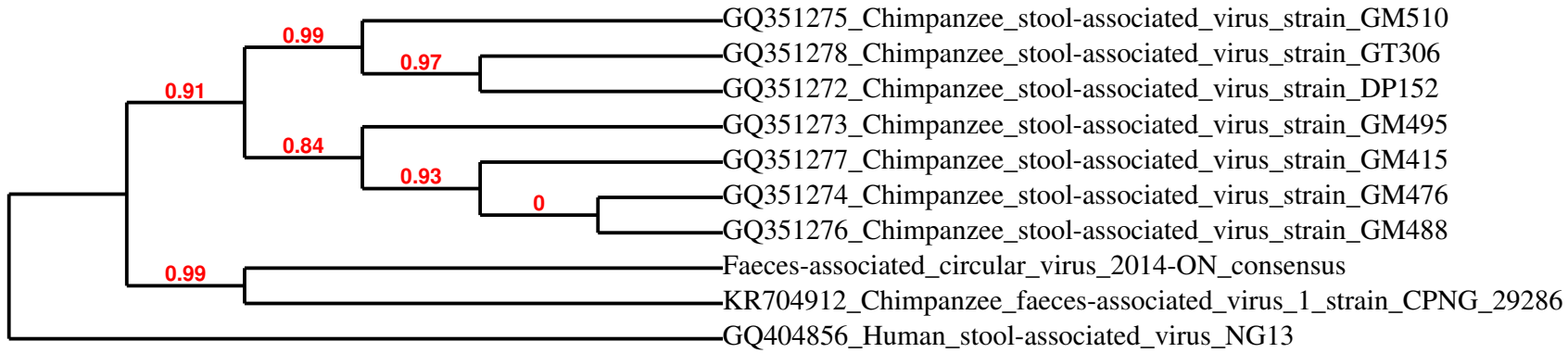
Table 3.6. Top 10 most prevalent non-vertebrate viral sequences detected in 67 samples.

Detected virus	Accession number	% of total non-phage sequences (2532)	Average identity (%)	Prevalence in samples (%)
Mimivirus terra2	KF527228	10	79	63
Megavirus courdo11	JX975216	8	82	58
Cafeteria roenbergensis virus BV-PW1	GU244497	7	82	24
White spot syndrome virus strain CN01	KT995472	5	73	19
Aureococcus anophagefferens virus isolate BtV-01	KJ645900	5	76	46
Chrysochromulina ericina virus isolate CeV-01B	KT820662	5	74	61
Tokyovirus A1	AP017398	2	79	40
Culex pipiens densovirus	FJ810126	1	69	30
Acanthocystis turfacea Chlorella virus 1	AY971002	1	71	18
Melanoplus sanguinipes entomopoxvirus	AF063866	1	82	18

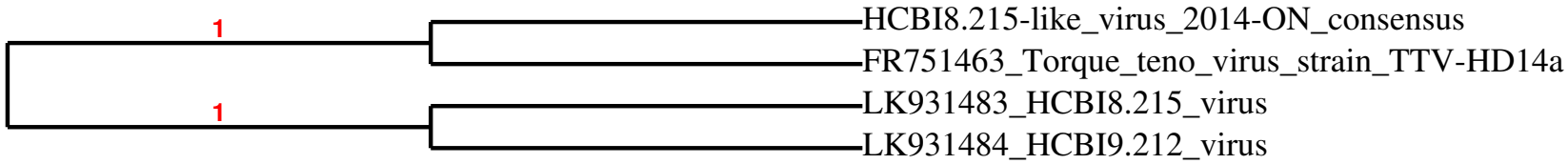
a


AF083424_Ateline_herpesvirus_3
X64346_Saimiriine_herpesvirus_2
Herpesvirus_2014-ON_consensus
AH002362.2_Varicella-zoster_virus
0.98

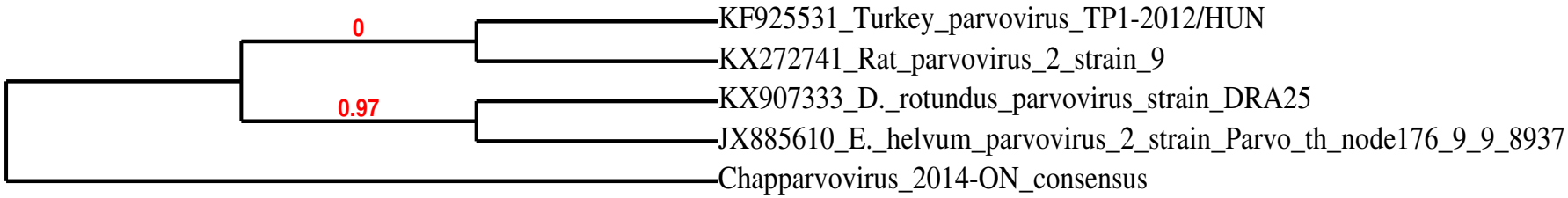
b


GQ351275_Chimpanzee_stool-associated_virus_strain_GM510
GQ351278_Chimpanzee_stool-associated_virus_strain_GT306
GQ351272_Chimpanzee_stool-associated_virus_strain_DP152
GQ351273_Chimpanzee_stool-associated_virus_strain_GM495
GQ351277_Chimpanzee_stool-associated_virus_strain_GM415
GQ351274_Chimpanzee_stool-associated_virus_strain_GM476
GQ351276_Chimpanzee_stool-associated_virus_strain_GM488
Faeces-associated_circular_virus_2014-ON_consensus
KR704912_Chimpanzee_faeces-associated_virus_1_strain_CPNG_29286
GQ404856_Human_stool-associated_virus_NG13
0.99
0.97
0.91
0.84
0.93
0
0.99

c


1
1
HCBI8.215-like_virus_2014-ON_consensus
FR751463_Torque_teno_virus_strain_TTV-HD14a
LK931483_HCBI8.215_virus
LK931484_HCBI9.212_virus

d


0
0.97
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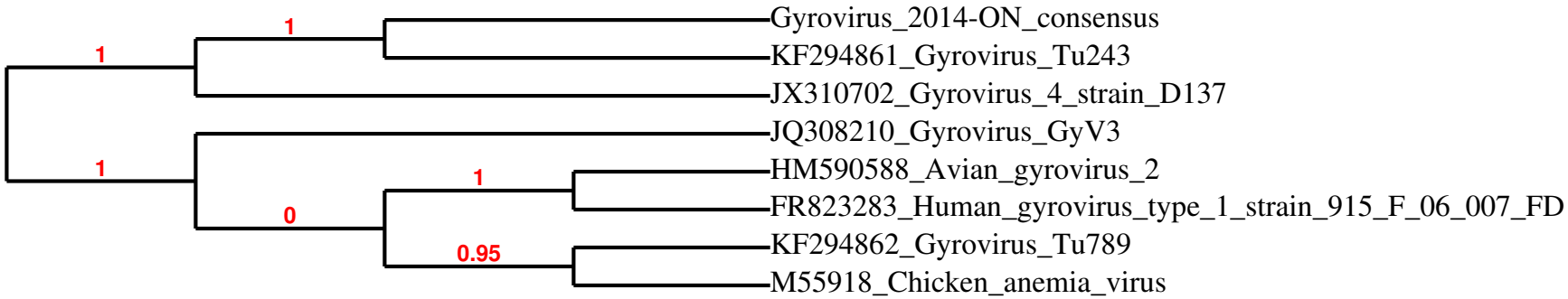
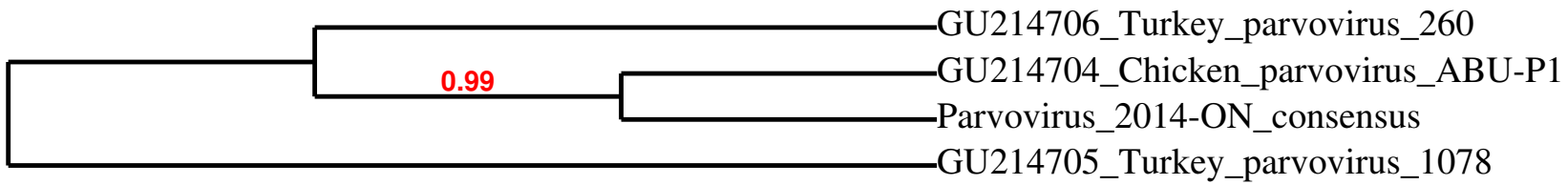
KX272741_Rat_parvovirus_2_strain_9
KX907333_D._rotundus_parvovirus_strain_DRA25
JX885610_E._helvum_parvovirus_2_strain_Parvo_th_node176_9_9_8937
Chapparvovirus_2014-ON_consensus

e


1
1
1
1
0
0.95
Gyrovirus_2014-ON_consensus
KF294861_Gyrovirus_Tu243
JX310702_Gyrovirus_4_strain_D137
JQ308210_Gyrovirus_GyV3
HM590588_Avian_gyrovirus_2
FR823283_Human_gyrovirus_type_1_strain_915_F_06_007_FD
KF294862_Gyrovirus_Tu789
M55918_Chicken_anemia_virus

f



g

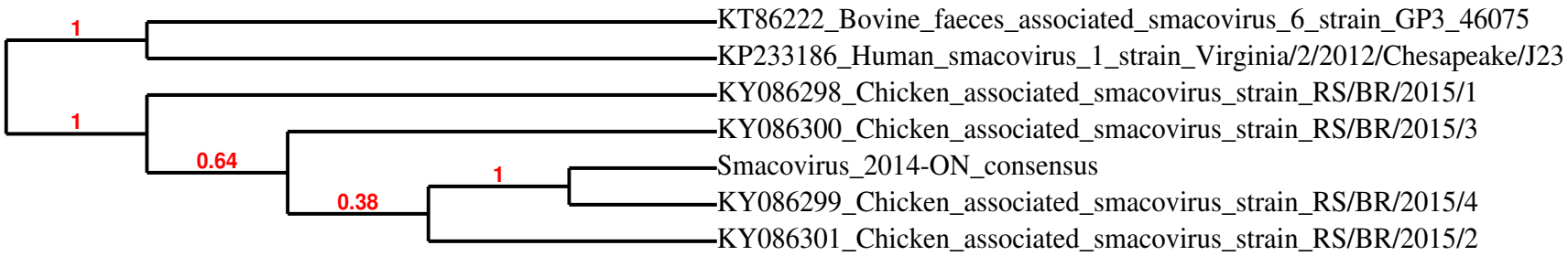


Figure 3.1. Phylogenetic comparison of consensus sequences to related viruses **a**. Herpesvirus 2014-ON_consensus **b**. Faeces-associated circular virus 2-14-ON_consensus **c**. HCBI8.215-like virus 2014-ON_consensus **d**. Chapparvovirus 2014-ON_consensus **e**. Gyrovirus 2014-ON_consensus **f**. Parvovirus 2014-ON_consensus **g**. Smacovirus 2014-ON_consensus

Chapter 4: General discussion

The present study used virus-specific PCR and metagenomic methods to study viral shedding in the feces of Ontario commercial mink. This included evaluating the prevalence of astrovirus, rotavirus and hepatitis E virus (HEV) in commercial mink over 4 years, with several significant findings regarding the detected viruses. Additionally, a diverse range of bacteriophage and eukaryotic virus sequences were detected in the fecal virome of farmed mink, with the first known report of mink bocavirus and rotavirus in Canada. This research has shown that age and diet likely play major roles in enteric viral populations, and that there are potentially zoonotic viruses shed in the feces of commercial mink.

4.1 The importance of evaluating rotavirus, astrovirus and hepatitis E virus prevalence in Ontario commercial mink

Previous research has shown that enteric astrovirus is widespread on commercial mink farms, playing a major role in causing mink diarrhea (De Benedictis et al., 2011; Englund et al., 2002; Mittelholzer et al., 2003). This study has shown that over four years (2014-2017), astrovirus was detected in 14% of samples overall with samples collected from kits and the summer season having significantly more positive samples. These results are consistent with previous research showing that kits are more susceptible to enteric astrovirus compared to adult female mink, and active infections are considered a significant risk factor for pre-weaning diarrhea (Englund et al., 2002; Mittelholzer et al., 2003).

Notably, the number of astrovirus-positive samples across the four sampled years decreased from 25% to 1% (2014 and 2017 respectively); however, this comparison may be skewed since only female samples were collected in 2017, and it has already been established that astrovirus shedding is more prevalent in kit samples. Although this may be attributable to the lack of kit samples collected in the winter cohort, it has also been shown that certain infections (mainly gram-negative bacteria) are more prevalent in the summer season compared to the winter season (Eber et al., 2011; Leekha et al., 2012; Zhang et al., 2016). The number of astrovirus-positive adult female samples from the summer cohort was significantly higher compared to samples from the winter cohort (2014-2015 and 2016-2017, $p=0.001$), which may imply that astrovirus shedding is more prevalent in the summer season regardless of the ages of the animals. Sequencing of astrovirus-positive samples showed that,

with the exception of mink AV 2017_ON_11az, all other sequences clustered closely with previously identified mink astrovirus strains at the RdRp gene. Since mink AV 2017_ON_11az was the only astrovirus positive sample detected from the 2017 cohort, it was not possible to determine whether other astrovirus sequences from the same cohort were closely related to the newly detected strain or previously reported strains.

In contrast to astrovirus, the proportion of HEV and rotavirus-positive samples remained fairly consistent across all sampled years. Although HEV has not been directly associated with clinical disease in mink, it has been suggested that co-infection of HEV with other viral agents could collectively contribute to disease (Krog et al., 2013). Interestingly, 7 samples from the summer cohort (2014 and 2015) were found to be HEV and astrovirus positive. Although these samples were collected from seemingly healthy animals, the health status of mink on farms is often not subject to veterinary surveillance and is therefore up to the interpretation of producers. Even among veterinarians, the definition of diarrhea or enteric disease may vary. In this sense, we cannot determine the exact health status of animals or the implications of potential viral infections. Similar to astrovirus, the number of HEV-positive samples was found to be significantly higher in kit samples compared to adult female samples, and in samples collected in the summer compared to the winter. Although we do not know if mink HEV plays a role in causing enteric disease, a correlation between HEV co-infections in mink and disease could help to characterize some diarrheal outbreaks with unknown etiological agents. All detected HEV sequences were closely related to previously reported mink HEV strains from Denmark, indicating that mink HEV is likely highly conserved across farms (Krog et al., 2013).

This is the first report of rotavirus-RNA detected in the fecal matter of commercial mink. In this study, the number of rotavirus-positive samples were fairly consistent across the years sampled, although this number seemed to be increasing. A significantly increased number of rotavirus-positive samples were detected in the 2017 cohort compared to the 2014 cohort (females only, $q=0.008$), as well as in the 2017 cohort compared to the 2016 cohort ($q=0.005$), although this increase was not as apparent. The number of rotavirus-positive samples overall was low relative to the number of astrovirus and HEV-positive samples. Rotavirus is associated with diarrheic disease in various species, particularly juvenile animals and humans (Bonica et al., 2015; Steyer et al., 2008). It may also be a zoonotic agent in commercial animals, with evidence showing highly related rabbit and bovine rotavirus

sequences, as well as a case study of a rabbit rotavirus isolated from a child (Bonica et al., 2015; Schoondermark-van de Ven et al., 2013). Interestingly, most of the sequences from rotavirus-positive samples detected in this study clustered closely with previously reported porcine rotavirus strains, indicating that the porcine products in the mink diet may have resulted in shedding of this virus. No significant differences in rotavirus shedding was found between age groups or season of collection, although the comparisons between age groups only accounted for samples collected in 2014-2015. Additionally, one rotavirus sequence detected in 2015 was not closely related to any other detected sequences (including one other sequenced 2015 sample) or previously reported strains of porcine and bovine rotaviruses. This may be due to a different source of rotavirus infection in the mink or due to contamination of the sequenced sample.

These results further confirm that virus shedding, which could correlate to infection, is more common in kits than in females. Diet may to play a role in the shedding of viruses with greater zoonotic potential (rotavirus and HEV). Season may also be a factor in the prevalence of astrovirus, rotavirus and HEV infections, although this remains to be determined. Seasonal differences as well as co-shedding of HEV and astrovirus were notable findings, but the definitive effects of these factors could not be determined. Further research is required to first determine if virus shedding reflects enteric infections. Sampling of both healthy and diseased kits and adult female mink in subsequent years would allow for better understanding of the influence of age group and season on viral infections, and the effect of co-infections on mink health. It must also be noted increased sampling on farms may result in higher detection rates of virus shedding, since only a small fraction of mink from each farm were sampled for this study.

4.2 The importance of characterizing the mink fecal virome and the implications of the detected viral sequences

Previous viral metagenomic studies in wild and domestic carnivores have identified a diverse range of eukaryotic virus as well as bacteriophage sequences (Bodewes et al., 2014; Conceição-Neto et al., 2015; Duarte et al., 2013; Fehér et al., 2014; Ng et al., 2014; Phan et al., 2011; Smits et al., 2013; Zhang et al., 2014). Previous fecal virome studies in ferrets and felids have found the most prevalent viral sequences to belong to the families *Parvoviridae*, *Anelloviridae* and *Picornaviridae*, *Astroviridae*, *Reoviridae*, *Hepeviridae*, *Papillomaviridae*,

Picobirnaviridae, and *Coronaviridae* (Fehér et al., 2014; Ng et al., 2014; Smits et al., 2013; Zhang et al., 2014). This mink fecal viral metagenomic study found the highest number of viral sequences from the families *Herpesviridae*, *Parvoviridae*, *Circoviridae*, *Anelloviridae* and *Picornaviridae*. Particular viruses of interest include mink bocavirus, which was first described in China by Yang et al. (2016), and has not yet been associated with diarrhea in mink. This study showed high prevalence of mink bocavirus with high identity to the previously reported bocavirus, indicating that this may be a relatively conserved virus. Sequences with similarity to saimiriine herpesvirus 2 and chimpanzee faeces-associated virus 1 CPNG_29286 had low average identity (71% and 70%, respectively), indicating that the detected sequences are from divergent viruses that may be specific to mink, or are contaminants from the time of collection. Two human gyroviruses (Tu243 and GyV3) were also identified. The detected sequences had high average identity to gyrovirus GyV3 (94%), and low identity to gyrovirus Tu243 (67%). Gyroviruses have been previously isolated from the skin of healthy humans and from the fecal samples of children with diarrhea, however it is unknown how these viruses would affect mink health (Phan et al., 2014; Tung G Phan et al., 2012). Finally, of the 15 prevalent vertebrate viruses described, 7 were avian-associated viruses. This is most likely due to the poultry based diet of commercial mink. Farmed mink are also fed a variety of porcine products, but of the most prevalent vertebrate viruses described, only sequences with high identity to posavirus 3 strain 958-4 (previously associated with nematodes parasitizing pigs) were detected. These results further demonstrate the need to assess to role of diet in viruses shed in the feces of domestic animals. In addition to the vertebrate viral sequences, water and soil-associated algal and amoebal viruses, insect and fungal viruses were also detected, which may be due to sample contamination, or are a part of the mink GI tract. These would not be expected to have a great effect on mink health.

Relative to the number of phage sequences, the number of eukaryotic virus sequences detected was low, making up 2% of all identified viral sequences >100 bp. Most other virome studies found the majority of identified sequences to be eukaryotic viruses, and only a small percentage of sequences matched with bacteriophage sequences (Bodewes et al., 2014; Smits et al., 2013; Zhang et al., 2014). One study by Phan et al. (2011) found 26,846 reads with matches to eukaryotic virus and 154,000 reads with matches to phage, but no explanation was provided by the authors as to why more phage sequences were detected. The difference between the abundance of phage and eukaryotic virus sequences could be attributable to amplification bias in NGS. However, the bacteriophage sequences in this study were used for

comparison with a mink fecal microbiome study, which included samples from the same 2014 cohort that was used in this study. *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Clostridium*, *Escherichia*, *Streptococcus* and *Pseudomonas* species were also found to be prevalent in the mink microbiome. *Pseudomonas*, *Clostridium*, *Escherichia*, *Streptococcus* and *Salmonella* bacterial species may be associated with disease in mink (Martínez et al., 2013; Wilson et al., 2015). These findings suggest that bacterial and viral infections may play a synergistic role in mink health, furthermore, the influence of phage on these bacterial populations is not well understood.

Comparisons between age and farm groups (**Appendix Figure B.1**) in this study showed no significant differences in virus shedding for the most prevalent eukaryotic viruses and bacteriophage described, with the exception of *Pseudomonas* phage, which was detected in more adult female mink samples compared to kit samples. Since the relationship between phage and their bacterial host populations is unclear, it could not be determined if adult female mink are more susceptible to *Pseudomonas* phage infections, or if these infections would result in more diseased adult female mink. Increased sampling of diseased mink, or mink fecal virome analysis using samples collected over more than one year may show more apparent differences. Overall, these results show that the number of viral sequences are fairly consistent between Ontario commercial mink. Finally, it must be noted that no mink astrovirus, HEV or porcine rotavirus sequences targeted in PCR were detected in the NGS results. This may again be due to the amplification bias previously described, leading to the amplification of other bacteriophage and eukaryotic virus sequences which were more prevalent prior to sequencing.

4.3 Implications of phage-encoded antimicrobial resistance genes in commercial mink and antimicrobial resistant bacterial isolates

In the phage sequences screened in this study, 5 *E. coli*-associated antimicrobial resistance genes (ARG's) were identified. Although highly conservative parameters were used to search for these ARG's, the results of previous research suggest that it is unlikely that the phage detected in this virome study carry functional genes that may confer antimicrobial resistance to the bacterial population (Enault et al., 2017). However, the use of antimicrobials has been shown to increase the frequency of phage-encoded ARGs that could be transferred to bacterial populations (Allen et al., 2011; Modi et al., 2014; Pedersen et al., 2009; Ross and

Topp, 2015). Since antimicrobial use on commercial mink farms is often not recorded or monitored by a veterinarian, the effect of antimicrobial use on the likelihood of phage-encoded ARG's is unknown in commercial mink.

Only samples from 2014 were assessed for phage-encoded ARGs using NGS, but no bacterial resistance data is currently available for fecal samples collected in this year. The available reported data on antimicrobial use on mink farms in this cohort did not correspond to the results of the phage-encoded ARG's in this study, although this could be due to under-reporting of antimicrobial use by producers. Resistance data for *E. coli* isolates in mink fecal samples collected in 2016 and 2017 samples showed that in 22 isolates, 32% were resistance to at least one of the 14 antimicrobials tested, two of which were detected in the phage-encoded ARGs (gentamicin and streptomycin). These results are not comparable to the sequencing results described in this study (different years sampled), but it does demonstrate that *E. coli*-associated antimicrobial resistance is mostly likely the most common on commercial mink farms. Additionally, challenging the *E. coli* population with gentamicin and/or streptomycin may increase the number of phage-encoded ARGs that could confer antimicrobial resistance, although this needs to be explored further.

4.4 Future directions

As previously discussed, the detected viral sequences may not reflect active enteric infections in the GI tract. This is because the viral sequences detected in the pooled fecal samples may have passed through the GI tract without infection or colonization, or are no longer active infections. As the fecal samples were collected from beneath the mink pens, contamination from the environment or other animals is also possible. A comparison of viral sequences detected in the feces and the viral sequences isolated directly from the gastrointestinal tract of the animals would help to determine the reliability of both methods. Additionally, there is always the possibility that the viruses detected in this study are due to contamination, as the fecal samples are collected from under the pens in open shed farms. This study only used samples collected from clinically healthy mink, based on the producers' interpretation of the animals' health status. Many of the viruses that have been identified in this study (through detection of sequences shed in the feces) have not been previously associated with disease in mink or have not been confirmed to be the etiological agent in diseased mink. Sampling of diseased commercial mink for PCR testing and sequencing of the

fecal virome would allow for comparisons between healthy and sick animals. Further optimization of the protocol used to amplify viral sequences prior to NGS amplification using different reagents may allow for more targeted amplification of eukaryotic viruses. The phage-encoded antimicrobial resistance genes identified could be cloned and tested for their ability to confer resistance to the commensal *E. coli* population.

The work described is the first evaluation of astrovirus, rotavirus, and HEV in healthy mink from Canada, and it is also the first characterization of the commercial mink fecal virome using NGS. The results of this study have provided further understanding of prevalent viral sequences detected in the feces of commercial mink in Ontario, however, further information is required to determine the relationship between active enteric viral infections and the results of the methods used in this study. The relationship between common viral infections and productivity on farms (which may include mink pelt quality, litter size, animal mortality) are of major concern to producers, and need to be assessed further. Finally, given the growing concerns regarding antimicrobial use in commercial animals and the unintended effects this may have on not only the bacterial populations, but also the phage populations, more work is required to better assess the risk of ARG transfer from phage to bacteria.

4.5 Summary and conclusions

1) Astrovirus was detected in both pooled mink kit and adult female fecal samples with the highest number of positive samples compared to rotavirus and HEV over the four sampled years, but this number appears to be decreasing. All detected astrovirus sequences used in phylogenetic analysis clustered closely with previously reported mink astrovirus strains, with the exception of the only astrovirus sequence detected in 2017. Kits had significantly higher numbers of astrovirus-positive samples compared to adult female samples.

2) Rotavirus shedding in fecal samples was less prevalent than astrovirus, but these numbers appear to be increasing over the years sampled. This is the first report of rotavirus shedding in commercial mink in Canada, and the sequences detected were mostly related to porcine rotavirus strains, with one sequence that was not closely related to other sequenced samples or previously described rotavirus strains.

3) Hepatitis E virus shedding in commercial mink samples remained constant over the four years sampled, and sequenced samples clustered closely with previously characterized mink HEV strains. Kits had significantly higher numbers of HEV-positive samples compared to adult female samples.

4) Diverse viral sequences were detected in this mink fecal virome study, with the most prevalent sequences from families *Herpesviridae*, *Parvoviridae*, *Circoviridae*, *Anelloviridae* and *Picornaviridae.*

5) Avian-associated viruses, a porcine-associated posavirus, two human gyroviruses, and mink bocavirus were most prevalent among the vertebrate viruses detected.

6) Sequences of a potentially novel chapparvovirus was detected, which did not cluster closely with other related parvoviruses, indicating that this could be a mink-specific parvovirus.

7) Of the bacteriophage sequences identified, those with *Bacillus*, *Clostridium*, *Enterococcus*, *Escherichia*, *Lactobacillus*, *Lactococcus*, *Proteus*, *Pseudomonas*, *Salmonella*, *Shigella*, *Staphylococcus*, and *Streptococcus* bacterial hosts were most prevalent.

8) *Pseudomonas* phage sequences were detected in significantly more adult female samples compared to kit samples. There were no significant differences in any other of the prevalent viral sequences between adult female mink and kit samples.

9) *E. coli*-associated phage-encoded antimicrobial resistant genes were the most commonly detected, which was also reflected in antimicrobial testing of *E. coli* isolates from fecal samples collected from subsequent years.

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APPENDIX A: COMPREHENSIVE VIROME RESULTS

Table A.1. All detected phage groups based on bacterial host and the top species identified (109,612).

Phage group	Sequences detected	Top species
Escherichia phage	17542	*Enterobacteria* phage phiEcoM-GJ1
Enterococcus phage	12470	*Enterococcus* phage EFDG1
Bacillus phage	7260	*Bacillus* phage B103
Staphylococcus phage	6510	*Staphylococcus* phage 6ec
Lactoccocus phage	6184	*Lactococcus* phage Tuc2009
Streptococcus phage	4779	*Streptococcus* phage phiARI0923
Pseudomonas phage	4753	*Pseudomonas* phage Pf3
Salmonella phage	4247	*Salmonella* phage 9NA
Clostridium phage	3319	*Clostridium* phage 39-O
Lactobacillus phage	2884	*Lactobacillus* phage phiJL-1
Proteus phage	2439	*Proteus* phage PM 75
Shigella phage	2389	*Shigella* phage pSf-1
Acinetobacter phage	2382	*Acinetobacter* phage LZ35
Geobacillus phage	2356	*Geobacillus* phage GBK2
Enterobacter phage	1886	*Enterobacter* phage Tyrion
Cronobacter phage	1766	*Cronobacter* phage Dev-CD-23823
Pseudoalteromonas phage	1762	*Pseudoalteromonas* phage H101
Arthrobacter phage	1741	*Arthrobacter* phage Mudcat
Listeria phage	1715	*Listeria* phage A006
Cellulophaga phage	1711	*Cellulophaga* phage phi10:1
Citrobacter phage	1448	*Citrobacter* phage CR8
Klebsiella phage	1368	*Klebsiella* phage F19
Idiomarinaceae phage	1302	*Idiomarinaceae* phage 1N2-2
Persicivirga phage	1261	*Persicivirga* phage P12024L
Flavobacterium phage	1242	*Flavobacterium* phage 11b
Paenibacillus phage	1215	*Paenibacillus* phage Tripp
Vibrio phage	1208	*Vibrio* phage N4
Erwinia phage	1203	*Erwinia* phage FE44
Edwardsiella phage	1196	*Edwardsiella* phage PEi21
Listonella phage	1193	*Listonella* phage phiHSIC
Mannheimia phage	1156	*Mannheimia* phage vB_MhS_1152AP2
Rhodococcus phage	1128	*Rhodococcus* phage ReqiPepy6
Mycobacterium phage	1111	*Mycobacterium* phage Bane1
Croceibacter phage	1102	*Croceibacter* phage P2559Y
Morganella phage	197	*Morganella* phage MmP1
Streptomyces phage	191	*Streptomyces* phage SF3
Yersinia phage	174	*Yersinia* phage Berlin
Rhodobacter phage	167	*Rhodobacter* phage RcRhea
Kluyvera phage	156	*Kluyvera* phage Kvp1

Weissella phage	146	*Weissella* phage WCP30
Pectobacterium phage	142	*Pectobacterium* phage PM1
Parabacteroides phage	137	*Parabacteroides* phage YZ-2015b
Polaribacter phage	128	*Polaribacter* phage P12002L
Leuconostoc phage	127	*Leuconostoc* phage Ln-9
Synechococcus phage	127	*Synechococcus* phage S-MbCM7
Aeromonas phage	124	*Aeromonas* phage pAh6-C
Brevibacillus phage	120	*Brevibacillus* phage Jenst
Chlamydia phage	117	*Gokushovirinae* Bog1183_53
Psychrobacter phage	117	*Psychrobacter* phage Psymv2
Erysipelothrix phage	84	*Erysipelothrix* phage SE-1
Serratia phage	62	*Serratia* phage Eta
Clavibacter phage	40	*Clavibacter* phage CMP1
Erwinia phage	13	*Erwinia amylovora* phage Era103
Iodobacteriophage	7	Iodobacteriophage phiPLPE
Verrucomicrobia phage	7	*Verrucomicrobia* phage P8625
Xanthomonas phage	1	*Xanthomonas* phage vB_XveM_DIBBI

Table A.2. All detected vertebrate viral sequences (1,237).

Vertebrate viral sequences	Sequences detected
Mink bocavirus clone 1	134
Posavirus 3 strain 958-4	134
Chimpanzee faeces associated circular DNA molecule 1 isolate CPNG_29268	119
Chicken anemia virus	84
Saimiriine herpesvirus 2 complete genome	71
Avian adeno-associated virus strain DA-1	56
Avian gyrovirus 2	54
HCBI8.215 virus complete sequence	48
Desmodus rotundus parvovirus strain DRA25	46
Gyrovirus Tu243	37
Chicken parvovirus ABU-P1	25
BeAn 58058 virus	24
Avian adeno-associated virus ATCC VR-865	24
Chicken associated smacovirus strain RS/BR/2015/4	22
Caribou feces-associated gemycircularvirus	21
Badger feces-associated gemycircularvirus strain 588t	21
Volepox virus strain CA	21
Ovine herpesvirus 2 strain BJ1035	19
IAS virus	18
Ungulate bocaparvovirus 6 strain USII/03	18
Fowl adenovirus D	17
Turkeypox virus strain TKPV-HU1124/2011	17
Porcine stool-associated circular virus 4 isolate CP2	14
Human herpesvirus 7	7
Bovine faeces associated circular DNA virus 1 isolate GP3-46075_cow2	6
Human herpesvirus 8	6
Sheeppox virus 17077-99	6
Deerpox virus W-848-83	5
Gyrovirus 4 strain D137	5
Porcine parvovirus	5
Turkey parvovirus 1078	5
Chicken stool-associated gemycircularvirus strain RS/BR/2015	5
Gyrovirus 4	4
Gyrovirus GyV3	4
Macacine herpesvirus 3	4
Penguinpox virus isolate PSan92	4
Rabbit fibroma virus	4
Raccoon dog amdovirus isolate HS-R	4
Bat mastadenovirus WIV12	4
Gyrovirus Tu789	4
Alcelaphine herpesvirus 1	4

Cyprinid herpesvirus 3	4
Parus major densovirus isolate PmDNV-JL	4
Alcelaphine herpesvirus 2 isolate topi-AlHV-2	3
Duck adenovirus 2 strain GR	3
Faeces associated gemycircularvirus 21 isolate 29_Fec80018_llama	3
Human herpesvirus 6A	3
Murine roseolovirus isolate YOK1	3
Pig stool associated circular ssDNA virus GER2011	3
Pigeonpox virus isolate FeP2	3
Rat stool-associated circular ssDNA virus isolate KS/11/0577	3
Protoparvovirus HK-2014 isolate ParvoQ45/2013	3
Badger feces-associated gemycircularvirus strain 588t	2
Bovine herpesvirus 5	2
Bovine herpesvirus 6 isolate Pennsylvania 47	2
Equid herpesvirus 4	2
Felis catus gammaherpesvirus 1 isolate 31286	2
Frog virus 3	2
Murid herpesvirus 1	2
Porcine circovirus 2	2
Skunkpox virus strain WA	2
Turkey stool associated circular ssDNA virus strain TuSCV	2
Macaca nemestrina herpesvirus 7	2
Orf virus	2
African swine fever virus strain BA71V	1
Avian paramyxovirus 2 strain APMV-2/Chicken/England/7702/06	1
Ball python nidovirus strain 07-53	1
Bat circovirus POA/2012/VI	1
Black howler monkey smacovirus isolate SF1	1
Cowpox virus	1
Duck faeces associated circular DNA virus 2 isolate 4_Fec60467_duck	1
Elephant endotheliotropic herpesvirus 4 isolate North American NAP69	1
Elephantid herpesvirus 1	1
Equid herpesvirus 1	1
Equid herpesvirus 8	1
European hedgehog papillomavirus	1
Goose circovirus	1
Gyrovirus GyV7-SF	1
Human adenovirus 54	1
Human adenovirus F	1
Human erythrovirus V9	1
Human genital-associated circular DNA virus-1 isolate 349	1
Human herpesvirus 3	1
Human herpesvirus 4	1

Human immunodeficiency virus 1	1
Human papillomavirus type 92	1
Human parainfluenza virus 1	1
Human respiratory syncytial virus	1
Magpie-robin coronavirus HKU18	1
Mouse cyclovirus isolate Cyclo-sf1	1
Muscovy duck circovirus	1
Porcine stool-associated circular virus 6 isolate XP1	1
Raccoonpox virus	1
Rat bocavirus strain HK1S	1
Saimiriine herpesvirus 4 strain SqSHV	1
Simian retrovirus 8 strain SRV8/SUZ/2012	1
Squirrel poxvirus strain Red squirrel UK complete genome	1
Turkey adenovirus 1	1
Turkey adenovirus 4 isolate TNI1	1
Turkey adenovirus 5 isolate 1277BT	1
Adeno-associated virus - 4	1
Adeno-associated virus 5	1
Bovine ephemeral fever virus	1
Bovine papular stomatitis virus strain BV-TX09c1	1
Yaba-like disease virus	1
Vaccinia virus	1
Variola virus	1
Tupaiid herpesvirus 1	1
Sumatran orang-utan polyomavirus complete genome, isolate Pi	1
Suid herpesvirus 1	1
Respiratory syncytial virus	1
Oropouche virus segment M	1

Table A.3. All detected non-vertebrate eukaryotic viral sequences, including water, soil, algae, insect, fungal, and crustacean-associated viruses (1,295).

Virus	Sequences detected
Mimivirus terra2	246
Megavirus courdo11	203
Cafeteria roenbergensis virus BV-PW1	169
Aureococcus anophagefferens virus isolate BtV-01	139
Chrysochromulina ericina virus isolate CeV-01B	129
White spot syndrome virus strain CN01	116
Tokyovirus A1	60
Culex pipiens densovirus	34
Acanthocystis turfacea Chlorella virus 1	30
Melanoplus sanguinipes entomopoxvirus	30
Choristoneura rosaceana entomopoxvirus 'L'	22
Paramecium bursaria Chlorella virus 1	20
Cedratvirus A11	16
Yellowstone lake mimivirus isolate: 1	15
Solenopsis invicta densovirus isolate SiDNV-Arg	9
Amsacta moorei entomopoxvirus 'L'	7
Adoxophyes honmai enomopoxvirus 'L'	6
Choristoneura biennis entomopoxvirus 'L'	4
Hubei picorna-like virus 50 strain spider113255	4
Anomala cuprea entomopoxvirus strain: CV6M	3
Junonia coenia densovirus	3
Lake Sarah-associated circular virus-10 isolate LSaCV-10-LSSO-2013	3
Grapevine virus F	3
Ectocarpus siliculosus virus 1	2
Hyposoter fugitivus ichnovirus segment C11	2
Golden Marseillevirus	1
Grapevine fleck virus	1
Hokovirus HKV1 Hokovirus_4	1
Hubei arthropod virus 3 strain GCM7473	1
Hubei sobemo-like virus 24 strain WHLC5390	1
Hubei tombus-like virus 18 strain QTM27278	1
Hubei virga-like virus 12 strain SCM49209	1
Mythimna loreyi densovirus	1
Narcissus symptomless virus	1
Niemeyer virus, partial genome	1
Okra yellow mosaic Mexico virus DNA B	1
Peanut chlorotic streak virus	1
Sulfolobus islandicus rudivirus 3 isolate SIRV3	1
Sulfolobus spindle-shaped virus 7	1

Sulfolobus turreted icosahedral virus	1
Wuhan house centipede virus 2 strain arthropodmix22554	1
Wuhan pillworm virus 3 strain WHSFII20254	1
Wuhan spider virus 8 strain spider134060	1
Xestia c-nigrum granulovirus	1
Zygosaccharomyces bailii CLIB 213	1

APPENDIX B: FARM GROUPINGS BASED ON GEOGRAPHICAL LOCATION IN ONTARIO

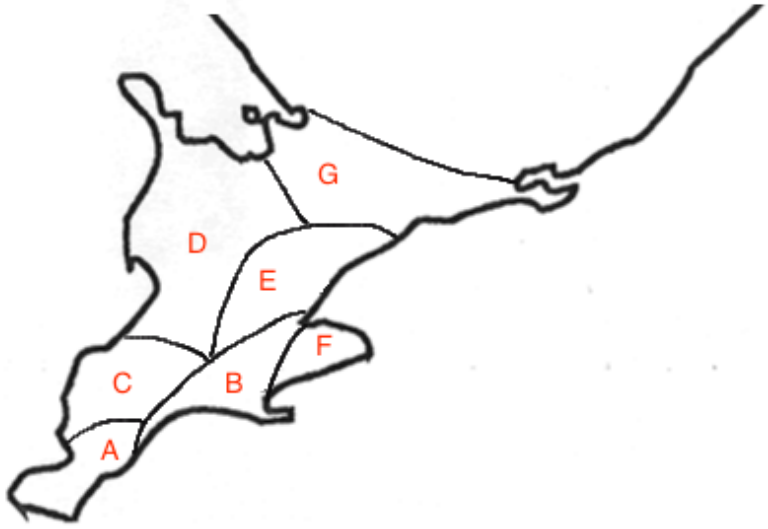

Figure B.1. Geographical grouping of 40 commercial mink farms in Ontario, where farms were grouped into 7 groups (A-G) based on geographical proximity to each other.

APPENDIX C: SWINE HEPATITIS E VIRUS SHEDDING IN ONTARIO MINK

C.1 Introduction

Porcine hepatitis E virus (HEV) has been shown to have significant zoonotic impact, and infection is spread though the consumption of undercooked pork products (Abravanel et al., 2017; Martin-Latil et al., 2016; Mykytczuk et al., 2017). Mink are often fed a mixed diet of pork offal, poultry and fish by-products – some raw and some cooked, however, there are no standard regulations in the production of mink feed. The results of this virome study have further shown that diet may play an important role in virus shedding in the feces of mink, but the prevalence of porcine HEV shedding has yet to be evaluated in commercial mink. Although mink HEV is thought to contribute to clinical disease in commercial mink, whether swine HEV plays a role in diseases in mink is unknown (Krog et al., 2013). Additionally, the presence of swine HEV on mink farms poses zoonotic risk to humans working proximity to the animals, since swine HEV is known to cause acute hepatitis in humans. Due to poor biosecurity on farms, monitoring of this potentially zoonotic virus may help to reduce the risk of infections on mink farms.

C.2 Materials and Methods

The previously described sampling, viral RNA extraction, and cDNA synthesis protocols were used for samples used for swine HEV testing of 527 pooled adult female mink and kit samples collected between 2014-2017. A 348 bp fragment of swine HEV open reading frame 2 (ORF2) was targeted in a nested PCR using primers 3156NF and 3157NR (5-AATTATGCYCAGTAYCGRGTTG-3' and 5'- CCCTTRTCYTGCTGMGCATTCTC-3' respectively, annealing temperature 55°C) for the first reaction, and primers 3158NF and 3159NR (5'-GTWATGCTYTGCATWCATGGCT-3' and 5'AGCCGACGAAATCAATT CTGTC-3' respectively, annealing temperature 55°C) for the second reaction (using 2 µL of product from the first reaction) (Banks et al., 2004; Gyarmati et al., 2007). The swine HEV positive controls were synthesized based on the primer targeted regions (gBlocks Gene Fragments, IDT). PCR products were visualized on 2% agarose gels with 12 µL of SyberSage and a 0.1-10.0 kb DNA ladder (New England Biolabs). The partial sequences of targeted swine HEV genes detected in samples were compared to existing NCBI reference sequences. A cladograms was constructed to compare the detected partial sequence to the most closely related NCBI reference sequences (based on % identity) for all sequenced genes

based on the maximum likelihood method (Dereeper et al., 2008).

C.3 Results

Of 527 tested samples, one adult female sample from the winter collection period of 2015 was positive for swine HEV (0.2%). **Figure C.1** shows the relationship between the sequenced swine HEV positive sample swine HEV_2015_ON_26e and closely related swine HEV strains P-D2-3a, 2014-018, 2014-033, P-D2-1a, P-D2-4a (KT778281, KX530975, KX530980, KT778280, respectively), which were isolated from Canadian swine herds. Swine HEV_2015_ON_26e also clustered closely with HEV strains Philippines-HEV-6-38, -41 and -43 (KF546259, KF546258, KF546257), which were isolated from a river in the Philippines. The detected sequence was not as closely related to swine HEV strains swSTHYVD505-L/2011/CA, swSTHYVB7120-L/2012/CA, 2014-035, 2014-014, D2-13b, and P-D2-2c (KF956540, KF956536, KX530981, KX530974, KT778293, KT778282, respectively).

C.4 Discussion

Swine HEV was only detected in one sample of the 527 tested, however it must be noted that, due to random sampling of only a small fraction of animals on farm, these results may not be fully representative of true swine HEV prevalence. The detected sequence was also highly related to other strains previously detected in Canadian swine herds, and could be the result of poor biosecurity on farm in feed practices or contamination from commercial animals in proximity to the mink pens (Krog et al., 2013). PCR testing of mink feed and increased sampling on farms may better reveal the source of the detected swine HEV, and provide more information on the true prevalence of swine HEV on commercial mink farms.

C.5 References

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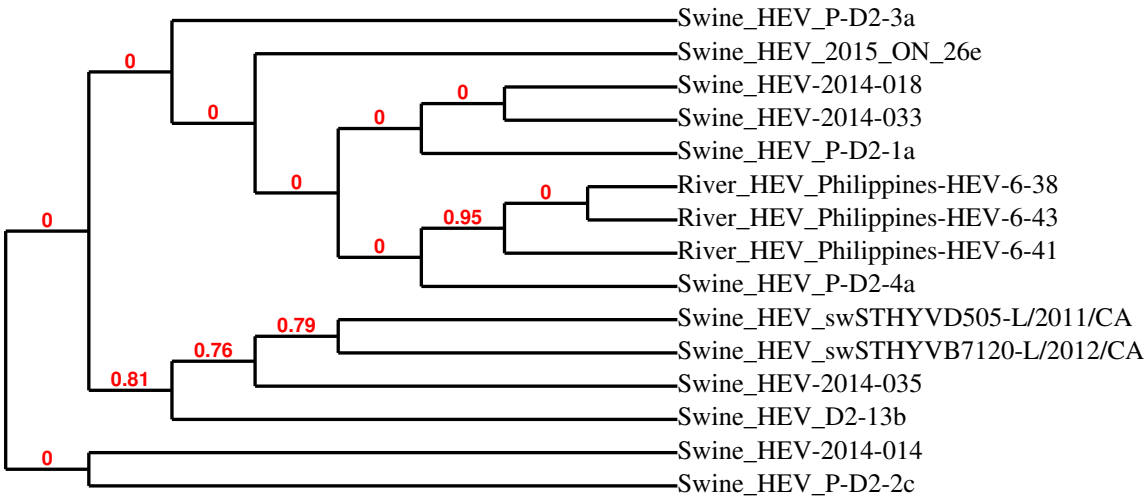

Figure C.1 Phylogenetic analysis of detected swine hepatitis E virus sequence swine_HEV_2015_ON_26e to closely related viruses