INFLUENZA VIRUSES FROM WILD BIRDS IN NEWFOUNDLAND AND LABRADOR IN THE CONTEXT OF GLOBAL INFLUENZA DYNAMICS

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Influenza viruses from wild birds in Newfoundland and Labrador in the context of global influenza dynamics

by

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Abstract

The primary hosts for avian influenza A viruses (AIV) are waterfowl and shorebirds, although other groups such as seabirds and gulls also serve as hosts. Newfoundland is an important breeding area for boreal and subarctic birds, and a wintering location for some high-latitude North American, and Eurasian species. I gathered 2873 samples from seabirds, gulls and waterfowl in Newfoundland and Labrador during 2008-2010. The overall detection rate of AIV in these birds was low, but viruses were identified in Common Murre (*Uria aalgae*), Thick-billed Murre (*U. lomvia*), American Black Duck (*Anas rubrpies*), Great Black-backed Gull (*Larus marinus*), and other unknown gull species. An AIV isolated from a Great Black-backed Gull in 2008 had segments with a mosaic pattern of geographical origins, indicating transatlantic transmission of AIV between Newfoundland and Europe. These findings, as well as analyses of six viruses sequenced from gulls in Alaska and all gull AIV sequences available in public databases, suggest that large gulls may play an important role in AIV dynamics, especially in the context of global movements.

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List of Abbreviations

AIV	Avian influenza A virus
cDNA	Complimentary single stranded DNA
HA	Hemagglutinin, RNA segment 4
HPAIV	High pathogenic avian influenza A virus
LPAIV	Low pathogenic avian influenza A virus
M	Matrix, RNA segment 7
NA	Neuraminidase, segment 6
NCBI	National Centre for Biotechnology
NL	Newfoundland and Labrador
NP	Nucleoprotein, RNA segment 5
NS	Non-structural protein, RNA segment 8
PA	Polymerase acidic protein, RNA segment 3
PB1	Polymerase basic protein 1, RNA segment 2
PB2	Polymerase basic protein 2, RNA segment 1
PCR	Polymerase chain reaction
RT-PCR	Reverse transcriptase polymerase chain reaction
rRT-PCR	Real time reverse transcriptase polymerase chain reaction
VTM	Viral transport media

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Chapter 1

Introduction

1.1 General Introduction

1.1.1 Disease emergence and influenza A virus

Infectious disease emergence and re-emergence in humans, domesticated animals and wildlife has been an prominent feature of the last century (Friend *et al.* 2001). The capacity of influenza A virus to undergo rapid antigenic transformation and re-emerge within a population makes it one of the most important re-emerging infectious diseases of the twentieth century (Webby and Webster 2001). Infectious diseases have the potential to play a significant role in the regulation, composition, and diversity of a species, particularly on a population level (Smith *et al.* 2009). Not only do infection and disease affect local populations, but these populations also play important roles in the introduction, geographical expansion, host-range expansion and re-emergence of disease (Friend *et al.* 2001; Si *et al.* 2009). It is through these factors that infectious diseases can transform from local to global phenomena (Friend *et al.* 2001).

Disease is conspicuous only when a number of animals die over a short time period and carcasses accumulate. As a result, interest has centered on animal diseases that cause mass-mortality events and on agents that cause conspicuous pathology (Wobeser 2006). Indeed, the first isolation of avian influenza virus (AIV) from wild birds occurred after a mass-mortality event (Becker 1966). Consequently, surveillance of apparently healthy wild birds to detect and characterize these viruses began, and it was discovered that there were large pools of influenza viruses in wild birds that did not cause obvious

morbidity or mortality (Alexander and Brown 2009). Subsequent surveillance and characterization of these low pathogenic AIV (LPAIV) has contributed to our understanding of the genetics, evolution, and overall ecology of these viruses.

1.1.2 *Influenza A structure and genetics*

Influenza viruses belong to the family *Orthomyxoviridae* (Kawaoka *et al.* 2005), and are divided into three genera, Influenza A, Influenza B, and Influenza C, based upon antigenic properties of the nucleocapsid (NP) and matrix (M), and structural variations (Webster and Kawaoka 1988). Wild birds are naturally infected only with influenza A viruses (Webster *et al.* 1992); thus only these AIV will be discussed further. The virion is enveloped, and spherical or pleiomorphic in shape (Webster *et al.* 1992). The genome consists of eight segments of unlinked, negative-sense, single-stranded RNA: PB2, PB1, PA, HA, NP, NA, M and NS (Webster *et al.* 1992; Kawaoka *et al.* 2005) that encode for 11 proteins (Webster *et al.* 1992; Chen *et al.* 2001). There are two main surface proteins, HA and NA, of which there are 16 and 9 different forms, respectively, all of which have been detected in wild birds (Webster *et al.* 1992; Olsen *et al.* 2006).

The different proteins have functions in entry (HA, M2), RNA replication (PB2, PB1, PA, NP), packaging (M1, NS2), exit from the host cells (NA, M1), and immune system evasion (NS) (Webster *et al.* 1992). Continued evolution of the two major surface proteins, HA and NA, is particularly important for continuing host immune system evasion, enabling the virus to successfully enter, replicate within, and exit the cells of the host.

1.1.3 Influenza A evolution

There are two main mechanisms through which AIV diversify. The first, referred to as antigenic drift, occurs due to an error-prone RNA-dependant RNA polymerase that lacks proofreading ability (Gething *et al.* 1980; Both *et al.* 1983; Webster *et al.* 1992). The second, referred to as antigenic shift, occurs due to co-infection and reassortment. Reassortment occurs due to the unlinked nature of the eight RNA segments, and thus if a cell is infected by more than one influenza virus, the progeny can contain various combinations of segments from the different original viruses (Gething *et al.* 1980; Webster *et al.* 1992) (Figure 1.1).

AIV have recently been demonstrated to have rapid rates of evolutionary change, characterized by accumulations of synonymous and nonsynonymous mutations (Chen and Holmes 2006; Chen and Holmes 2010). However, the time of origin of the circulating PB2, PB1, PA, NP and M segments is recent, approximately 100-130 years ago, compared to 516, 1097 and 1256 years ago for the NS, NA, and HA segments, respectively (Chen and Holmes 2010). It has been hypothesized that occasional selective sweeps in the HA and NA genes, with transient linkage to the other segments occurs, resulting in the shallow history of these currently circulating AIV segments (Chen and Holmes 2010).

Geographical segregation of host species has resulted in two different, and independently evolving, lineages of AIV genes: American and Eurasian (Donis *et al.* 1989; Webster *et al.* 1992). Gene flow between the two lineages occurs but is infrequent (Obenauer *et al.* 2006; Krauss *et al.* 2007; Dugan *et al.* 2008; Bahl *et al.* 2009; Chen and Holmes 2009). Intercontinental exchange, or the introduction of segments between

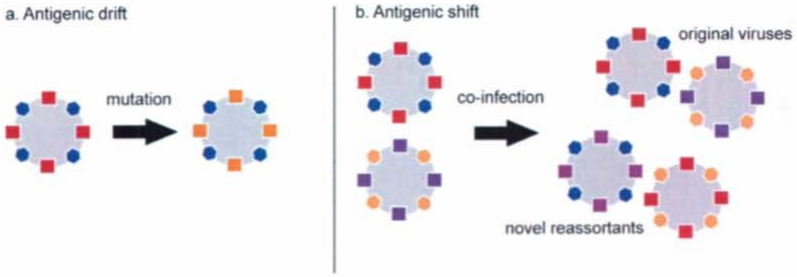

Figure 1.1 Mechanisms of evolution of AIV. a. Antigenic drift. The error-prone nature and lack of proof-reading ability of the RNA polymerase result in mutations in the RNA sequence during replication, and therefore altered protein sequences. b. Antigenic shift. Reassortment of the unlinked RNA segments can occur if a cell is co-infected by more than one virus. Viral progeny can include both novel reassortants, which contain some combination of segments from both parental viruses, or viruses with the same composition of segments as the parental strains. All eight AIV segments are subject to both antigenic shift and antigenic drift, but the apparent mutation and reassortment rates are greatest in the HA and NA segments due to immune pressure, and selection for functionality in the other segments.

geographic regions, occurs mainly from Eurasia to America. All currently circulating lineages of the PB1 and PA segments in North American wild aquatic birds are derived from Eurasian introductions that occurred at some time in the 1960s, which has lead to the exclusion of endemic versions of these segments in North America (Bahl *et al.* 2009). To date, the intercontinental exchange of a complete genome has never been detected (Krauss *et al.* 2007; Dugan *et al.* 2008). As a result, the overall evolution of AIV is influenced by mutation, reassortment, genetic linkage, natural selection, and population subdivision (Chen and Holmes 2010).

1.1.4 AIV in wild bird reservoirs

AIV are transmitted and maintained through the fecal-oral route, where the virus enters the bird through the oral or nasal passages, replicates in the intestinal cells, and is excreted in the feces (Webster *et al.* 1992). Disease transmission is dependant on biotic factors such as host-agent adaptations, susceptibility of the host, and threshold host densities, in addition to abiotic factors such as temperature, pH, and salinity (Webster *et al.* 1992; Wobeser 2006).

The predominant viruses isolated from wild bird hosts are LPAIV, but HPAIV also occur. Wild birds are thought to be the reservoirs of all subtypes of AIV (Hinshaw *et al.* 1980; Webster *et al.* 1992), and AIV have been isolated from at least 105 species in 26 different families (Olsen *et al.* 2006). However, viruses are most frequently detected in Anseriformes (ducks, geese, swans) and Charadriiformes (shorebirds, terns, gulls) due to increased emphasis in these groups. Dabbling ducks, particularly Mallards (*Anas platyrhynchos*), have accounted for the most LPAIV isolations globally (Olsen *et al.*

2006). Among migratory wild birds, prevalence varies greatly according to species, year, season, and location. Generally, AIV infection in ducks is highest in the fall when large numbers of immunologically naïve birds are congregating prior to migration (Hinshaw *et al.* 1985). In contrast, peak prevalence of AIV in shorebirds is during the spring, specifically at large migratory stop-over locations, such as Delaware Bay, during the migration towards northern breeding areas (Krauss *et al.* 2004). These differences in AIV prevalence are attributed to differences in migratory and feeding behaviour, habitat preferences, and geographic ranges (Stallknecht and Brown 2007).

Migratory birds are able to move pathogens around the globe, providing infection does not negatively affect body condition and migratory ability. Birds infected with LPAIV generally show no apparent clinical signs (Webster *et al.* 1992; Olsen *et al.* 2006). However, it has been demonstrated that LPAIV infections can affect body health and migratory ability (van Gils *et al.* 2007; Latorre-Margalef *et al.* 2008), however the effect of LPAIV infection on wild birds is still poorly understood. Conversely, poor body health and poor migratory performance may contribute to an increased susceptibility of AIV infection (Flint and Franson 2009).

Unlike LPAIV infection, HPAIV infection often results in significant morbidity and mortality of the infected bird host (Webster and Rott 1987). The first AIV detected in wild birds was HPAIV isolated from the Common Tern (*Sterna hirundo*) in South Africa during a mass mortality event (Becker 1966). Mechanistically, the switch from LPAIV to HPAIV is due to changes in the HA protein. The introduction of basic amino acid residues to the cleavage site in the precursor hemagglutinin protein (HA0, which is cleaved into HA1 and HA2 portions during virus maturation) results in enhanced viral

replication outside the respiratory and intestinal tracts because of increased HA cleavability (Alexander 2000). The subtypes H5 and H7 have accounted for most HPAIV isolations in wild birds (Olsen *et al.* 2006; Alexander 2007). The switch from LP to HP forms occurs after the introduction of these LP H5 and H7 into poultry (Alexander 2000), but this switch has never been documented in wild bird hosts (Alexander 2000; Alexander 2007). HPAIV has been isolated predominantly from domestic gallinaceous birds (chickens, turkeys, quail) (Alexander 2000; Perkins and Swayne 2001), and there has only been one isolation of HPAIV that could not be associated with outbreaks of HPAIV in poultry (Becker 1966; Alexander 2007). The HPAIV H5N1 virus that appeared in Asia in the 1990s has affected wild birds, the poultry industry, wild bird markets, and domesticated waterfowl in Eurasia and Africa, and continues to circulate in these areas (Gauthier-Clerc *et al.* 2007; Alexander and Brown 2009). HPAIV H5N1 outbreaks have been linked to both poultry exchange and wild bird migratory patterns, but are presently contained within Eurasia and Africa (Kilpatrick *et al.* 2006). HPAIV H5N1 has been isolated in wild bird stop-over locations in Eurasia, such as Novosibirsk, Russian Republic, and Lake Qinghai, China (Wang *et al.* 2008). Due to the spread of HP H5N1 within the old-world, there has been great concern that wild birds will carry this virus to North America (Peterson *et al.* 2007), possibly through Alaska (Winker and Gibson 2010).

1.1.5 AIV in gulls and seabirds

AIV studies in waterfowl have completely overshadowed work with other species by number, variety and distribution (Alexander 2007). In the United States between 2006

and 2008, 83% of AIV surveillance samples were collected from dabbling ducks, which are a behavioural-ecologically defined group of birds that inhabit freshwater shallows which feed by tipping tail-up to reach aquatic plants, seeds and snails including *Anas* spp (Pedersen *et al.* 2010). Due to this, our understanding of AIV in other species, such as seabirds and gulls, is poor.

Seabirds include all species in the order Procellariformes, some families of the Charadriiformes, and some families of the Pelecaniformes. They are a behavioural-ecologically defined group of birds capable of spending their lives at sea, some species staying relatively close to shore and others wandering the entire oceans in search of food, but all capable of living for extended period independent of land (Gaston 2004). It is important to note that some gull species meet the definition criteria as seabirds (e.g., Black-legged Kittiwake *Rissa tridactyla*), while others are plastic and live as seabirds in only some parts of their range, or at some times of year (e.g., Great Black-backed Gull *Larus marinus*) (Gaston 2004; Olsen and Larsson 2004). For reasons of clarity, I refer to gulls separately from seabirds, although overlap may occur. Due to intercontinental, pelagic and intracontinental movement, gulls and seabirds may be important in the movement of AIV around the globe. Indeed, it has been determined that intercontinental AIV segment exchange occurs more frequently in the Charadriiformes than other host groups (Makarova *et al.* 1999; Dugan *et al.* 2008; Kishida *et al.* 2008; Bahl *et al.* 2009), however the contributions of specific families and species within the order are still unclear.

As mentioned above, although waterfowl and shorebirds appear to be the primary avian reservoir, gulls and seabirds are also AIV hosts (Olsen *et al.* 2006). Prevalence of

avian influenza is low in both gull (<0.1%-13%) and seabird (<0.1%-4.26%) species (Olsen *et al.* 2006; Krauss *et al.* 2007; Munster *et al.* 2007; Ip *et al.* 2008; Velarde *et al.* 2010). However, very little sampling effort is placed upon these groups of birds, with only 4% of samples collected in the United States from 2006-2008 coming from gulls, terns and auks (Pedersen *et al.* 2010). Our understanding of seabird AIV is particularly poor due to low surveillance efforts, few virus identifications, and fewer available genome sequences. At the time I started my work, sequence information was only available from three viruses isolated from murres (*Uria* spp.) globally (Wallensten *et al.* 2005; Obenauer *et al.* 2006), and seven viruses from shearwaters (*Puffinus* spp.) in West Australia from 1970-1985 (Downie *et al.* 1973; Downie *et al.* 1977; Mackenzie *et al.* 1984). More sequence information is available from AIV isolated in gulls compared to seabirds, although gull virus dynamics are still not clear. It has been determined that gulls carry two unique HA subtypes, H13 and H16 (Hinshaw *et al.* 1982; Fouchier *et al.* 2005), and are more likely to have segments with a mosaic pattern of geographic origins (Dugan *et al.* 2008). Gull-specific clades of viruses have been identified (Olsen *et al.* 2006), but there is no evidence that these viruses constitute a separate gene pool (Kawaoka *et al.* 1988; Hanson *et al.* 2008). Gull and shorebird viruses are often grouped together (Kawaoka *et al.* 1988; Hanson *et al.* 2008), and gull viruses have not been analyzed as an independent group.

1.1.6 Newfoundland and Labrador as an important breeding, foraging and mixing location for seabirds and gulls

Newfoundland and Labrador is home to the most important concentration of breeding seabirds in eastern North America (Gaston and Jones 1998), with tens of millions of seabird pairs (Cairns *et al.* 1989). On Baccalieu Island (aprrox 7 km^2 in size) alone there are 3.5 million pairs of breeding Leach's Storm Petrels (*Oceanodroma leucohora*) (Cairns *et al.* 1989). Abundant small pelagic schooling fish, especially capelin (*Mallotus villosus*), as well as favorable colony sites, make the various islands of Newfoundland and Labrador an ideal breeding area (Gaston and Jones 1998). Newfoundland breeding seabirds arrive from rich offshore winter foraging areas in the western North Atlantic Ocean, Gulf of Mexico, and coast of Africa (Gaston and Jones 1998; Mowbray 2002; Gaston *et al.* 2008). Additionally, tens of millions of seabirds winter in the vicinity of Newfoundland and Labrador and depart in spring to breeding grounds in the Canadian arctic and subarctic, Greenland, Iceland, and northern Europe (Gaston 2004). As a result, large numbers of various seabird species with different life-history strategies, from numerous geographic locations mix along the coast of Newfoundland. These dense mixing areas may be important in the transmission of diseases, such as AIV between species or hemispheric gene pools.

A large summer breeding seabird population, in addition to open landfill sites and traditionally large fisheries, also results in very large breeding and wintering gull populations (Drury 1973; Drury 1974; Blokpoel and Tessier 1986; Roy 1986; Good 1998; Robertson *et al.* 2001; Veitch 2003). Species such as Great Black-backed Gull and American Herring Gull (*L. smithsonianus*) not only breed on the island of Newfoundland,

but also maintain large wintering populations on the island that likely include local breeders and birds originating elsewhere (Pierotti and Good 1994; Good 1998). Some Newfoundland breeding individuals also migrate southward along the Atlantic coast (Gaston *et al.* 2008). Ring-billed Gulls (*Larus delawarensis*) breed in large numbers and winter in small numbers in eastern Newfoundland, the origin of the wintering individuals being unclear (Ryder 1993). In addition to Great Black-backed Gulls and American Herring Gulls, Arctic-breeding gull species such as Glaucous Gull (*L. hyperboreus*), Iceland Gull (*L. glaucoides*) and the occasional Ivory Gull (*Pagophila eburnea*) also winter in Newfoundland (Gilchrist 2001; Mallory *et al.* 2002; Snell 2002). Furthermore, presumably because it is the most eastern landmass in North America, Eurasian gull species overwinter on the island as well, especially Black-headed Gull (*Chroicocephalus ridibundus*) and Lesser Black-backed Gull (*L. fuscus*) (Olsen and Larsson 2004). Other Eurasian species occur as vagrants (on average one or two individuals wintering at the St. John's landfill and raw sewage outlets, e.g., Common Gull (*L. canus*), European Herring Gull (*L. argentatus*) and Yellow-legged Gull (*L. michahellis*) (Olsen and Larsson 2004). Of particular interest are recent records in St. John's of two gull species endemic to Japan and the Russian far east, the Slaty-backed Gull (*L. schistisagus*) and the Black-tailed Gull (*L crassirostris*), indicating an Asia-Pacific origin of some Newfoundland vagrant gulls. The timing of the movements of these wintering species often overlap on the island of Newfoundland during spring and fall months suggesting that Newfoundland has the potential to be an important area for transmission of AIV within and among all gull species.

1.2 Thesis overview

The gathering in Newfoundland and Labrador of seabird and gull species with wide geographic ranges presents a unique opportunity to study AIV in these under-studied hosts. Focusing on these hosts will help shed light on the role that species with pelagic and intercontinental migration patterns could have in both intra- and intercontinental AIV movements. The types of viruses and frequency of AIV intercontinental reassortment within these hosts could have implications in future surveillance for the potential entry and invasion of HPAIV segments into North America.

In the thesis, I explore the differences of AIV in a well described host family (ducks), a pelagic group (seabirds), and a family that spends time both in terrestrial and pelagic environments (gulls), and is known to associate with both ducks and seabirds. The goal of my thesis work was to determine which seabird and gull species in NL carry AIV, the prevalence of AIV in these species, how prevalence is related to seasonality, and to characterize the identified viruses in comparison with viruses isolated from local duck populations. Briefly, Chapter 2 describes the major findings pertaining to host species, spatial and temporal prevalence of AIV in seabirds, gulls and ducks in Newfoundland and Labrador. One of the viruses isolated from a Great Black-backed Gull in Newfoundland is characterized in detail and compared with other available AIV sequences in Chapter 3. Finally, an analysis of 6 viruses I isolated and sequenced from Alaska, in addition to other published sequences available in public databases, is presented in Chapter 4 to look at our current understanding of AIV in gull species globally.

1.3 Co-authorship Statement

Chapters 3 and 4 are versions of co-authored manuscripts that have been or will be submitted for publication, as detailed below. I wrote the original drafts of these manuscripts, which were then modified in response to the co-authors' comments.

Additionally, Chapter 2 contains results from a co-authored manuscript (Granter *et al.* 2010) and the Honours thesis of Ashley Dobbin (Dobbin 2009). Alissa Granter and I completed the sequencing of A/Thick-billed Murre/Newfoundland/031/2007(H11N2) collaboratively, and she completed the initial characterization of the virus for her Honours thesis. Ashley Dobbin characterized the M, NS, NP, and HA segments of the ducks viruses described in Chapter 2 and partially sequenced A/American Black Duck/Newfoundland/826/2008(H4) for her Honours thesis.

Manuscripts associated with work presented in this thesis:

Chapter 2 Granter A, Wille M, Whitney H, Robertson GJ, Ojkic D, Lang AS (2010). The genome sequence of an H11N2 avian influenza virus from a Thick-billed Murre (*Uria lomvia*) shows marine-specific and regional patterns of relationships to other viruses. **Virus Genes** 41:224-230

Chapter 3 Wille M, Whitney H, Robertson GJ, Ojkic D, Lang AS (2010). Reassortment of American and Eurasian genes in an influenza A virus (H13N2) isolated from a Great Black-backed Gull (*Larus marinus*), a species demonstrated to move between these regions. **Archives of Virology**. In press. DOI 10.1007/s00705-010-0839-1.

Chapter 4 Wille M, Robertson GJ, Whitney H, Runstadler J, Bishop MA, Lang AS. Extensive geographic mosaicism in avian influenza viruses from gulls in the northern hemisphere. **PLoS Pathogens**. In Review.

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Chapter 2

Influenza A viruses in selected wild birds of Newfoundland and Labrador, 2008-2010

2.1 Introduction

Influenza A viruses, in the family *Orthomyxoviridae*, are enveloped and possess a genome consisting of eight unlinked segments of negative-sense single-stranded RNA (Webster *et al.* 1992; Kawaoka *et al.* 2005). Influenza A viruses have dynamic evolutionary capabilities, with changes occurring through mutations, genome segment reassortment after co-infection by more than one virus, as well as the capacity to move between host species (Webster *et al.* 1992; Rambaut *et al.* 2008; Salomon and Webster 2009).

Wild birds are believed to be the reservoir for all subtypes of avian influenza virus (AIV) (Webster *et al.* 1992). Most identified strains of AIV are low pathogenic (LP), which are carried without obvious clinical signs. However, high pathogenic (HP) forms do occur, which cause significant morbidity and mortality in most bird hosts (Alexander 2007). AIV have been isolated from at least 105 wild bird species across 26 different families, with the highest frequency of sampling and prevalence of infection in *Anseriformes* (ducks, geese, and swans). Virus isolations from wild ducks overshadow isolations from all other host species by number, variety, and distribution (Alexander 2007). For instance, in the United States between 2006-2008, 83% of all AIV surveillance samples were collected from dabbling ducks; only 4% of samples were collected from gulls, terns and auks (Pedersen *et al.* 2010). Exploration of viruses from a broader host range with more emphasis on seabirds and gulls will contribute to our understanding of

AIV dynamics. Evidence suggests that viruses isolated from gulls posses unique characteristics (Obenauer *et al.* 2006; Dugan *et al.* 2008), including two hemagglutinin (HA) subtypes not found in viruses from other avian taxa (Hinshaw *et al.* 1982; Fouchier *et al.* 2005). There is very little information available pertaining to AIV in seabirds due to low surveillance effort, apparent low viral prevalence, and low priority for characterization of the viruses that have been found. As a result, our current understanding of low pathogenic avian influenza pertains to infection and viruses in ducks and shorebirds.

Newfoundland is an internationally important breeding location for various seabird species, particularly in the family *Alcidae* (Cairns et al. 1989). Additionally, it supports a large and diverse assemblage of wintering seabirds (Lock et al. 1994). Seabirds and most gulls do not follow the classic waterfowl flyways, but rather exhibit a movement from inshore breeding areas in the summer months to offshore foraging areas in the winter (Gaston and Jones 1998; Olsen and Larsson 2004). The offshore foraging patterns are very diverse across seabird species. Some move to local offshore regions, such as some species in the family *Alcidae* (Brown 1985), while others move across hemispheres, such as the Northern Gannet (*Moras bassanus*) (Mowbray 2002; Gaston 2004). In addition to being an important breeding location for seabirds, a unique assemblage of gull species is found in Newfoundland in winter, especially in St. John's. This is because of traditionally large fisheries, open landfill sites and raw sewage outfalls (Drury 1973; Drury 1974; Blokpoel and Tessier 1986; Robertson *et al.* 2001). In addition to local breeding species, St. John's urban wintering gull populations include two abundant Arctic gull species (Glaucous *Larus hyperboreus* and Iceland Gull *L.*

glaucoides) and numbers of two European species (Lesser Black-backed *L. fuscus* and Black-headed Gull *Chroicocephalus ridibundus*) (Gilchrist 2001; Snell 2002; Olsen and Larsson 2004). There is a period during the spring and fall when overwintering and summer breeding species, arriving from the various locations along the eastern seaboard of the United States of America (Gaston *et al.* 2008), can both be found in the same foraging and roosting locations. Numerous influenza viruses have been detected in gulls from Delaware in the spring and early summer (Bao *et al.* 2008), and some of these individuals may be breeding in Newfoundland. The co-occurrence of numerous species in Newfoundland from broad geographic ranges provides a unique opportunity to potentially detect a diverse array of influenza viruses.

Here I describe the results from testing 2873 samples collected from birds in NL over 2 consecutive years (May 2008-June 2010, in addition to stored carcasses from a murre wreck in 2007). Specifically, patterns in AIV prevalence pertaining to host species, seasonal variation and annual variation, and brief discussion of the viruses characterized are included.

2.2 Methods

2.2.1 Bird Sampling

A diversity of seabird species were targeted based upon results from other studies (Downie *et al.* 1973; Downie *et al.* 1977; Wallensten *et al.* 2005; Olsen *et al* 2006; Ip *et al.* 2008), the results from previous surveillance in Newfoundland (Table 2.1), and accessibility to seabird species in the province. Additionally, local breeding and winter resident gull species were targeted, as well as the local duck population. A target of 1000

Table 2.1 Avian influenza screening results from seabirds breeding on Gull Island[1], Newfoundland and Labrador, during the summer of 2006[2].

Species[3]	Total number of samples	Positive samples		Month
		RT-PCR	Isolation	
Atlantic Puffin	12	1	0	August
Leach's Storm Petrel	288	17	0	July - September
Black-legged Kittiwake	143	7	2	August
Great Black-backed Gull	169	6	5	July
American Herring Gull	296	31	17	July

[1] Coordinates provided in Appendix 3

[2] Completed by the Animal Health Division of Newfoundland and Labrador and the Canadian Cooperative Wildlife Health Centre.

[3] Scientific names provided in text.

live bird samples was set for each year, and all dead seabird, gull and duck carcasses were additionally sampled. The opening of the cloaca and the oropharyngeal cavity, were swabbed with sterile tipped applicators, which were inserted into a tube containing Multitrans viral transport media (VTM) (Starplex Scientific, Etobicoke, Canada). Oropharyngeal swabs were not collected from live birds in 2008. All live birds sampled for AIV were banded. Fecal samples were collected from roosting sites of gulls, with specific locations being selected based upon consistency of use, use by only gull species, and, if possible, use by only a single species of gull. Additionally in 2010, fecal samples were collected from ledges used by breeding Common Murres (*Uria aalge*). Only moist, freshly deposited fecal samples were collected to help ensure they were from the targeted species. Tubes containing samples were kept cool and placed at -80°C within 48 hours of collection.

2.2.2 Sample screening and virus culture

Sample screening and virus isolation were performed at the Animal Health Laboratory, University of Guelph. RNA was extracted from an aliquot of each swab sample using the MagMAX-96 Viral RNA Isolation Kit (Ambion, Streetsville, Canada) with an elution volume of 50 µl. Samples were assayed for the presence of the avian inflluenza matrix gene by real-time RT-PCR using the QuantiTect Probe RT-PCR Kit (Quagen, Mississauga, Canada) with a cutoff threshold (Ct) > 35.

Virus isolation was carried out in 9 to 11 day old SPF embryonated chicken eggs (Charles River, North Franklin, Connecticut) inoculated via the allantoic route. The eggs

were then candled daily to monitor for embryo mortality. Two blind passages were performed, and allantoic fluid was tested for hemagglutinating activity after each passage.

2.2.3 *RNA isolation and amplification of virus gene segments*

Allantoic fluid was mixed with an equal volume of TriPure Isolation Reagent (Roche, Mississauga, Canada) and RNA was extracted using the MagMax AI/ND Viral RNA Isolation Kit (Ambion) following the manufacturer's instructions. If the detected virus was not successfully cultured in allantoic fluid, and the Ct value was below 25, RNA was extracted from the original swab sample mixed with an equal volume of TRIzol LS Reagent (Invitrogen, Burlington, Canada). cDNA was synthesized using the Uni12M primer (Chan *et al.* 2006) (Appendix 1), which is complementary to a conserved sequence at the 3' ends of all AIV segments, and the Superscript III First Strand Synthesis System for Reverse Transcriptase PCR (Invitrogen) following the manufacturer's recommendations. Twenty-eight PCR reactions were then carried out in order to amplify the entire genome from this cDNA using a combination of previously published primers (Zou 1999; Phipps *et al.* 2004; Bragstad *et al.* 2005; Chan *et al.* 2006; Obenauer *et al.* 2006; Li *et al.* 2007; Koehler *et al.* 2008; Qiu *et al.* 2009) (Appendix 1). PCR products were purified using the QIAquick PCR Purification Kit (Qiagen). Capillary sequencing of PCR products was carried out at The Centre for Applied Genomics (Toronto, Canada). If non-specific amplicons were present, PCR products were cloned using the pGEM-T Easy Vector System (Promega, Madison, WI), and the appropriately sized cloned inserts sequenced using the M13 primers that bind to the plasmid vector.

Complete segment sequences were assembled using Geneious v3.8.5 (Biomatters, New Zealand).

2.2.4 Sequence analyses

Sequences were aligned using ClustalW version 1.4 and the resulting alignments used to construct a sequence similarity matrix within MEGA 4.0 (Tamura *et al.* 2007) to determine the percent (%) identities between NL sequences. The relationships of NL viruses to previously characterized viruses were investigated through BLAST searches of the GenBank sequence database. (Altschul *et al.* 1997)

2. 3. Results and Discussion

2.3.1 Sample collection

During this study 2873 samples were collected from 10 species of seabirds in 3 families, 8 species of gulls and 4 species of ducks in NL (Table 2.2). Samples were primarily collected from Atlantic Puffin (*Fratercula arctica*), Common Murre, Thick-billed Murre (*U. lomvia*), Razorbill (*Alca torda*), Leach's Storm Petrel (*Oceanodroma leucohora*), Black-legged Kittiwake (*Rissa tridactyla*), American Herring Gull (*Larus smithsonianus*), Great Black-backed Gull (*L. marinus*), and American Black Duck (*Anas rubripes*). In addition, samples were oppportunistically collected from Black Guillemot (*Cepphus grylle*), Dovekie (*Alle alle*), Northern Gannet, Manx Shearwater (*Puffinus puffinus*), Northern Fulmar (*Fulmarus glacialis*), Glaucous Gull, Iceland Gull, Ring-billed Gull (*L. delawarensis*), Black-headed Gull (*Chroicocephalus ridibundus*), Mallards

(*A. platyrhynchos*), Northern Pintails (*A. acuta*), American Black Duck x Mallard hybrids, and American Black Duck x Mallard x Domestic duck hybrids (Table 2.2).

Samples were collected from numerous islands along the east coast of Newfoundland, however seabirds samples were predominately collected from the Witless Bay Ecological Reserve and gull and duck samples from St. John's, Newfoundland (Figure 2.1, Appendix 2).

Host species, spatial, and temporal biases in the data set were caused by a number of factors such as limited accessibility to remote breeding locations, the locations of seabirds at sea during the winter months, poor weather conditions, and specific behaviour of seabirds in relation to reproductive success and predation. Preliminary AIV prevalence data from previous years were also used to inform sampling priorities. The low numbers of samples from certain host species such as Northern Gannets, which are numerous at two major breeding colonies, were due to the difficulty in accessing individuals. In contrast, low numbers of samples collected from other species, such as Manx Shearwater are due to inaccessability and low numbers of individuals in NL. A large number of samples were collected in a brief period to incorporate specific host species during specific time frames in locations that were logistically challenging to access, such as the Gannet Islands. Other locations, such as Gull Island and Great Island were consistently visited throughout this study, and therefore the reported AIV prevalence in seabirds are representative of these islands. Data from 2008 and 2009 are almost exclusivly from summer months, June–August, whereas sample collection in 2010 was more consistent across time.

Table 2.2 AIV surveillance in Newfoundland and Labrador between May 2008 and June 2010[1].

Family	Species	Prevalence								
		2007[2]		2008[3]		2009		2010[4]		Total
		AIV/ Effort[5]	%	AIV/ Effort[5]	%	AIV/ Effort[5]	%	AIV/ Effort[5]	%	%
Alcidae	Atlantic Puffin	0		0/125	0	0/114	0	0		0
	Common Murre	0		0/75	0	0/315	0	**1/70**	**1.4**	**0.2**
	Thick-billed Murre	**2/59**	**3.4**	0/27	0	0/278	0	**1/34**	**2.9**	**0.8**
	Razorbill	0		0		0/134	0	0		0
	Black Guillemot	0		0		0/1	0	0		0
	Dovekie	0		0/1	0	0		0		0
Procellariidae	Leach's Storm Petrel	0		0/201	0	0/117	0	0		0
	Northern Fulmar	0		0/2	0	0/2	0	0		0
	Manx Shearwater	0		0		0/12	0	0		0
Sulidae	Northern Gannet	0		0		0/27	0	0		0
Laridae	American Herring Gull	0		0/115	0	0/275	0	0/193	0	0
	Great Black-backed Gull	0		**2/31**	**6.5**	**3/33**	**9.1**	**2/108**	**1.9**	**4.0**
	Glaucous Gull	0		0/2	0	0/10	0	0/9		0
	Iceland Gull	0		0		0/14	0	0/4		0
	Ring-billed Gull	0		0/5	0	0		0		0
	Unknown gull	0		0/2	0	**2/26**	**7.7**	0/55	0	**2.4**
	Black-headed Gull	0		0/1	0	0		0		0
	Black-legged Kittiwake	0		0/81	0	0		0		0

Anatidae	**American Black Duck**	0	**10/180**	**5.6**	**6/78**	**7.7**	0		**6.2**
	Mallard	0	0/17	0	0/8	0	0/1	0	0
	American Black Duck -Mallard-Hybrids	0	0/4	0	0/6	0	0/1	0	0
	Northern Pintail	0	0/12	0	0/8	0	0		0

[1] AIV samples were collected from May 2008-June 2010, however some bird caracasses were collected in 2007.

[2] Influenza samples collected in May 2008, carcasses of birds collected in 2007.

[3] Surveillance from May-November 2008.

[4] Surveillance from January – June 2010.

[5] The number of positive samples by real time PCR (Ct<35)/the total number of samples screened from birds that year.

Host species from which AIV was detected in specific years are highlighted in bold face.

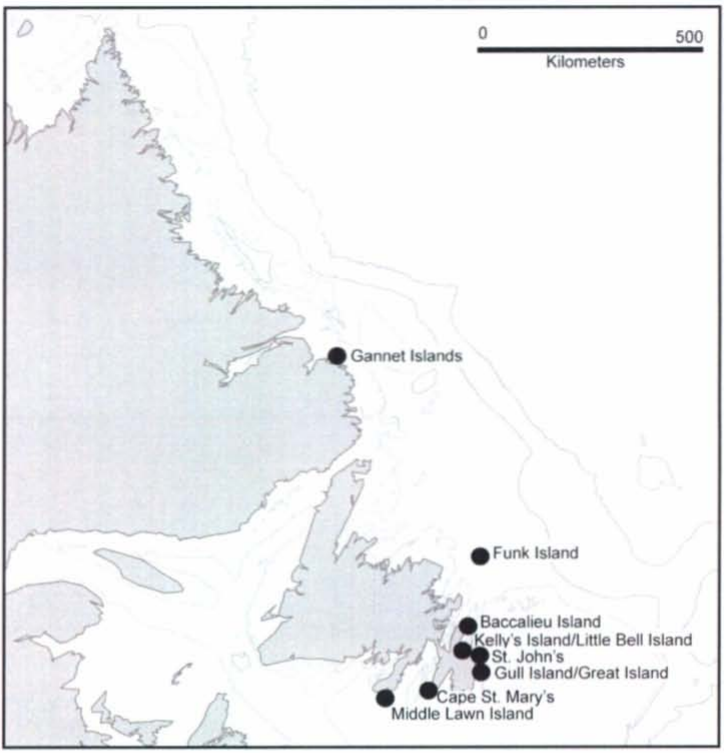

Figure 2.1 Locations in Newfoundland and Labrador from which AIV samples were collected from live birds. Specific locations in St. John's, and coordinates for all locations are listed in Appendix 6.

AIV were identified only from Thick-billed Murre in 2007 and 2009, 1 Common Murre in 2010, Great Black-backed Gull in all years, unknown gull species in 2009, and American Black Duck in 2008 and 2009 (Table 2.2). Specific trends in data collection and AIV prevalence in these species will be disucssed in detail below.

No viruses were detected from bird species within the *Procellariidae* or *Sulidae* families. Leach's Storm Petrels and Manx Shearwater, as well as Atlantic Puffins, are burrow-nesting seabirds that breed in dense colonies. Each burrow is only occupied by a pair of birds and their single chick (Huntington *et al.* 1996; Lee and Haney. 1996; Lowther *et al.* 2002). Due to this specific breeding behaviour, transmission of AIV is likely limited in these species. Further, a total of 13 samples were collected from Manx Shearwater on a single day, therefore the sample size, geographic scale and temporal scale were likely to limited to detect any AIV. Similarly, a total of 27 samples were collected from Northern Gannets, and therefore sample size was likely to low to detect any viruses. Species from which AIV were not detected in this study will not be disucssed further (Table 2.2)

Results from this study cannot be directly compared to results from the AIV survey in 2006 (Table 2.1). The samples collected in 2006 were screened at a different facility using different laboratory protocols. Therefore, the definition of "positive" and "inconclusive" may be different from those in this study (where a positive samples contains a Ct<35, and inconclusive sample contains a Ct >35 but less than <40). Further, these samples were unavailable, and therefore could not be incorperated into this study after re-screening.

2.3.2 Seasonal, temporal and spatial trends in AIV prevalence in Common and Thick-billed Murre

Common and Thick-billed Murres of all age groups were sampled from breeding areas in the summer months and opportunistically during the winter months (dead birds only; mass mortality events and hunter-killed birds). Common Murres breed on both Gull Island and Great Island, thus large numbers of samples were collected from both of these locations. Thick-billed Murres breed at low densities in a few locations along the coast of Newfoundland (Cairns *et al.* 1989), therefore the only samples collected during the summer months were from the Gannet Islands in Labrador. Both of these species spend the winter on both inshore and offshore Newfoundland waters; however, surveys from the murre hunt suggest there are more Thick-billed Murres compared with Common Murres during the winter months along the eastern coast of the island of Newfoundland (Elliot 1991; Gaston and Robertson 2010). Therefore, samples collected opportunistically from dead birds in the winter were predominantly from Thick-billed Murres. Carcasses sampled during the winter months were from birds that died during mass mortality events (2007 and 2009) (McFarlane Tranquilla *et al.* 2010) or that were shot during the murre hunt (all years). No samples were collected during the fall (Figure 2.2).

A total of four AIV were identified in murres, three of which were from Thick-billed Murres (Appendix 4). Overall prevalence of AIV in Thick-billed Murres in Newfoundland was low (0.8%) with the highest observed in 2007 (3.4%) (Table 2.2). A/Thick-billed Murre/NL/031/2007(H11N2) and A/Thick-billed Murre/NL/040/2007 were detected from birds collected during a mass mortality event on the Northeast Avalon Peninsula in 2007 (McFarlane Tranquilla *et al.* 2010). These carcasses were surveyed in

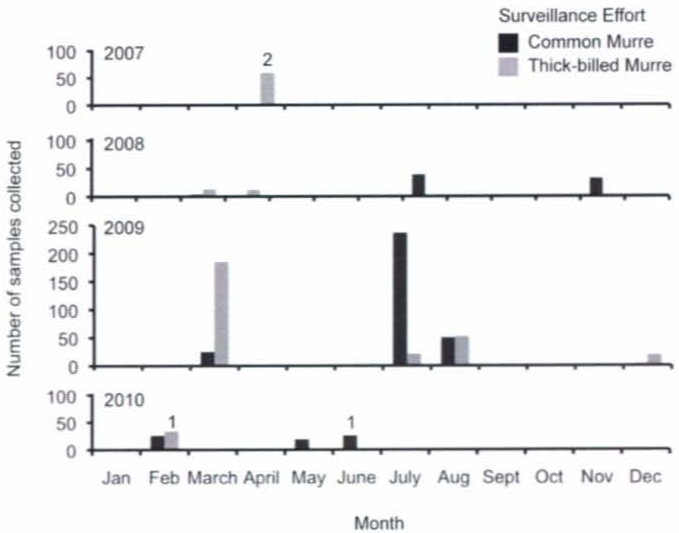

Figure 2.2 AIV surveillance effort and virus prevalence in Common and Thick-billed Murres in Newfoundland and Labrador. The numbers of samples collected are represented by columns, and the numbers of AIV detected are indicated by numbers. The dates of collection correspond to either carcass collection or bird capture.

2008, after having been frozen at -20°C for over 12 months. A/Thick-billed Murre/NL/MW108 was identified in a juvenile bird shot during the recreational hunt in St. Mary's Bay in February 2010 (Appendix 4). Viruses were only found in Thick-billed Murres between February and April, suggesting a seasonal trend. There were also differences between years, and no viruses were found in 2009 despite the large number of carcasses sampled in March 2009 (Figure 2.2).

Only one virus was detected from a Common Murre, in June 2010, suggesting low prevalence in this species (Table 2.2). Due to the low numbers of Common Murres in the vicinity of the Avalon Peninsula in the winter months, the number of samples collected from Thick-billed Murres overshadows the number from Common Murres. Therefore, the apparently low prevalence in Common Murres in relation to Thick-billed Murres may be caused by a seasonal sampling bias, rather than a genuine host-species trend. AIV detected in Murres in Alaska (Ip *et al.* 2008) and Sweden (Wahlgren *et al.* 2008) were from the summer months (July-August). The preliminary seasonal patterns in Newfoundland murres suggest that late winter and early spring could be primary times for AIV infection in these bird species. Continued and more consistent surveillance, and more viruses detected in murres through 2010-2011 will be important to resolve annual patterns and seasonal peaks in AIV prevalence.

These viruses represent the first seabird viruses detected in the Atlantic Ocean. AIV also has been detected in Thick-billed Murre in Alaska (Ip *et al.* 2008), whereas there has been a more global distribution of detections in Common Murres, including Sweden (Foucher *et al.* 2003; Wallensten *et al.* 2005), Russia (Sazonov *et al.* 1977) and

Alaska (Ip *et al.* 2008). Detection of viruses from both Common Murres and Thick-billed Murres across such a large geographic range suggests endemism, even if prevalence was low, although it is also possible the apparent low prevalence in murres reflects poor spatial and temporal sampling globally.

2.3.3 Seasonal, temporal and spatial trends in AIV prevalence in gulls

Great Black-backed Gull and American Herring Gull were the primary species of gulls targeted in this study, although small numbers of samples from various other species were also included as possible. Samples were primarily collected from breeding locations, such as Gull Island, Little Bell Island, Kelly's Island and Logy Bay, from both chicks and adults during the summer months. Small numbers of samples were collected in the remainder of the year through a combination of active capture of live birds, carcass collection, as well as collection of fresh fecal samples from gull roosting locations. Gull species congregate within St. John's city limits due to food availability from an open landfill site, therefore collection during the winter months occurred within the city limits. AIV were only found in Great Black-backed Gull. Viruses were identified in both direct sampling from carcasses, as well as fresh fecal samples collected from a winter roosting location. Two viruses were also detected in fecal samples for which the gull species were uncertain (Appendix 4). Prevalence of AIV in Great Black-backed Gulls in Newfoundland, 4.1%, is well within the range of AIV prevalence in gulls globally (0.1%-13%) (Olsen *et al.* 2006; Krauss *et al.* 2007; Munster *et al.* 2007; Ip *et al.* 2008; Velarde *et al.* 2010). Prevalence was highest in 2009 (9.1%) and lowest in 2010 (1.9%). I attribute

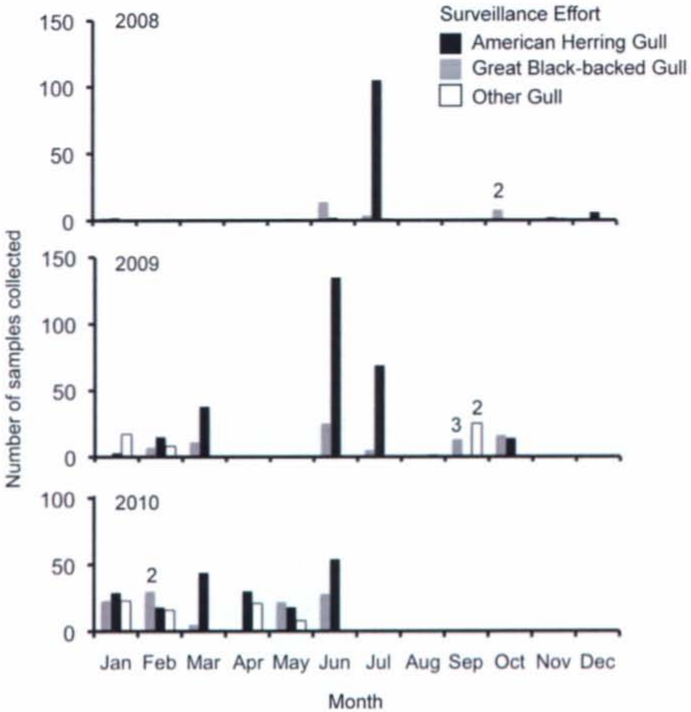

Figure 2.3 AIV surveillance effort and virus prevalence in gulls in Newfoundland and Labrador. Surveillance effort is represented by columns and the number of AIV detected are indicated by numbers. "Other gulls" includes Glaucous Gulls, Iceland Gulls, Ring-billed Gulls, Black-headed Gulls and fecal samples from locations with mixed species of gulls.

the low AIV prevalence in 2010 to seasonality, because in both 2008 and 2009 all viruses were detected in samples collected in the fall (September - October). The AIV detected in 2008 were from birds that died due to aspergillosis, a fungal infection caused by *Aspergillus fumigatus*. Viruses detected in 2009 and 2010 were in fresh fecal samples collected at Great Black-backed Gull roosting locations. Additional AIV were detected in fecals samples collected at a site used by both American Herring and Great Black-backed Gulls and at another site used by Ring-billed and American Herring Gulls (Appendix 4).

In North America, most of the AIV previously isolated from gulls were from American Herring Gulls and Laughing Gulls in Delaware and New Jersey (Bao *et al.* 2008). The number of American Herring Gull samples collected in NL surpassed that for Great Black-backed Gulls throughout spring and summer months, although similar numbers of samples were collected from both species in the fall (2009) and the winter (2009 and 2010) (Figure 2.3). Regardless, no AIV were identified in samples collected directly from American Herring Gulls, although American Herring Gulls may have been contributors to fecal samples for which the hosts were uncertain (Appendix 4). The apparent absence of AIV in American Herring Gulls in NL could reflect ineffective sampling over the required spatial or temporal scales, or that American Herring Gulls are simply infrequent hosts of AIV in NL.

AIV were not detected from Iceland Gull, Glaucous Gull, Ring-billed Gulls or Black-headed Gulls. This is likely due to small sample size, as well as restricted sampling temporally. These species are visitors to St. John's during the winter months, and therefore no samples were collected from these species during the fall, when most viruses were detected in Great Black-backed Gulls.

2.3.4 Seasonal, temporal and spatial trends in AIV prevalence in ducks

Samples from ducks were primarily collected in the fall (September - November) of 2008 and 2009. Not only was there an existing project to capture and mark ducks in the city at these times, but also prevalence in waterfowl is greatest in the fall when ducks are staging due to high densities of immunologically naïve young birds (Olsen *et al.* 2006). The timing of peak AIV prevalence during the fall collection period in St. John's is late September and early October. However, only a small number of samples were collected in the winter (2009 only) and samples were not collected in the summer months, therefore it is difficult to gauge the actual AIV prevalence outside of the fall in NL.

All American Black Ducks, Mallards, Northern Pintails and hybrid ducks captured in 2008 were sampled. American Black Duck prevalence in 2008 was 5.6%, and no viruses were identified in Mallards, Northern Pintails or hybrids (Table 2.2). Due to the large bias in viral prevalence in 2008, only American Black Ducks were targeted in the fall of 2009. Globally, the largest numbers of AIV isolations are from Mallards. In North America, 12.9% of 15250 samples contained AIV (Olsen *et al.* 2006). Therefore, it is surprising that no viruses were detected in this species, even with low effort. Northern Pintails are also important hosts in global AIV dynamics as they have high fall prevalence of AIV (11.2%) (Olsen *et al.* 2006), and they are one of the few duck species from which viruses containing segments from a mosaic of geographic origins have been isolated [in Alaska (Koehler *et al.* 2008; Ramey *et al.* 2010) and Japan (Liu *et al.* 2004)]. There is currently little information pertaining to AIV in American Black Ducks. Most studies including this host species were completed between 1975-1980, but combined prevalence from these 6 studies indicate that AIV was detected in 18.1% of American Black Ducks (Olsen

et al. 2006). Of the 130 viruses reported from American Black Ducks (as of 2006) (Olsen *et al.* 2006), there were only a total of 9 sequenced viruses in the GenBank database (as of July 2010).

Another interesting finding was that, in 2008, viruses were only found in birds from Mundy Pond, an important breeding area for American Black Ducks. No viruses were isolated from ducks in Quidi Vidi Lake, which contains one of the largest populations of ducks in the city year-round, or from Commonwealth Pond, which has a transient population of ducks. These findings are due to a combination of factors related to characteristics of the pond soil, water, and vegetation composition, the ecology and behaviour of the ducks in St. John's, Newfoundland, or ineffective sampling at the other locations. In 2009, samples were only collected from American Black Ducks at Mundy Pond.

All viruses, with the exception of A/American Black Duck/Newfoundland/1153/2009 were isolated from hatch year, or juvenile, birds. Furthermore, all viruses were isolated from male birds, with the exception of two in 2009 (Appendix 4). More samples were collected from hatch year, male, American Black Ducks in both 2008 and 2009, therefore it is possible that the observed AIV bias towards hatch year male birds was caused by sampling bias. Alternatively, immature male American Black Ducks infected with AIV may have been easier to capture (Flint and Franson 2009).

2.3.5 Characterization of AIV from Newfoundland

Only one seabird virus, A/Thick-billed Murre/Newfoundland/031/2007(H11N2), and one gull virus, A/Great Black-backed Gull/Newfoundland/296/2008(H13N2) were successfully sequenced and characterized. All viruses found in ducks in 2008, with the exception of A/American Black Duck/Newfoundland/865/2008(H4) and A/American Black Duck/Newfoundland/747/2008, were sequenced and characterized. These viruses will be briefly discussed.

The viruses isolated from the murre and gull were different from all of the duck viruses and from each other (Figure 2.4). Due to limited available AIV sequence information from seabirds, especially murres, it is not possible to ascertain the role that these hosts play in virus movement and evolution. However, available sequence information from Common Murres in Alaska and Sweden (Wallensten *et al.* 2005) suggest that viruses isolated from these hosts contain segments with a mosaic of geographic origins. All segments from A/Thick-billed Murre/Newfoundland/031/2007(H11N2) were similar to viruses isolated from North American waterfowl. A detailed discussion of this virus was provided by Granter *et al.* (2010) and is beyond the scope of this chapter. A detailed discussion of the phylogeographic patterns of A/Great Black-backed Gull/Newfoundland/296/2008(H13N2) is provided in Chapter 3. All segments for all duck viruses characterized were most similar to viruses isolated in North America. The M, NS and HA segments of all viruses were most similar to each other, however there were two different lineages of the PB2, PB1, and PA segments, and three different NP segment lineages (based on 99% identity criteria) (Figure 2.4). The two different lineages

Virus ID	Subtype	Segment							
		PB2	PB1	PA	HA	NP	NA	M	NS
031	H11N2								
296	H13N2								
812	H2								
836	H2N4								
840	H2N4								
732	H3								
734	H3N8								
807	H4N4								
826	H4								
819	H4N6								

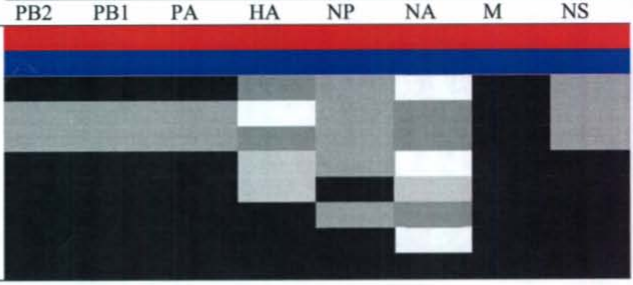

Figure 2.4 The genome constellations of AIV characterized from Newfoundland from 2007-2008. Identical colours by column represent sequences that are ≥99% identical, and different colours represent viruses that are <99% identical. White indicates no sequence is available. Newfoundland virus names are abbreviated to the within-year sample ID where 031 is A/Thick-billed Murre/Newfoundland/031/2008(H11N2), 296 is A/Great Black-backed Gull/Newfoundland/296/2008(H13N2), 812 is A/American Black Duck/Newfoundland/812/2008(H2), 836 is A/American Black Duck/Newfoundland/836/2008(H2N6), 840 is A/American Black Duck/Newfoundland/840/2008(H2N6), 732 is A/American Black Duck/Newfoundland/732/2008(H3), 734 is A/American Black Duck/Newfoundland/734/2008(H3N8), 807 is A/American Black Duck/Newfoundland/807/2008(H4N4), 819 is A/American Black Duck/Newfoundland/819/2008(H2N6), 826 is A/American Black Duck/Newfoundland/826/2008(H4).

of PB2, PB1 and PA contained the same viruses. However, the NP lineages contained different groupings of viruses (Figure 2.4). Viruses with consistently similar segments of the PB2, PB1, PA, NP, M, and NS included A/American Black Duck/Newfoundland/840/2008(H2N4) and A/American Black Duck/Newfoundland/836/2008(H2N4), A/American Black Duck/Newfoundland/819/2008(H2N6) and A/American Black Duck/Newfoundland/826/2008(H4) indicating transient linkage of these segments in this population of birds. The other duck viruses were reassorted between lineages (Figure 2.4). A more in-depth discussion pertaining to the HA, NP, M and NS segments of these duck viruses can be located in Dobbin (2009).

These data suggests high viral diversity and frequency of reassortment in American Black Ducks in Mundy Pond. This phenomenon has been demonstrated by other studies (Hatchette *et al.* 2004; Spackman *et al.* 2005; Dugan *et al.* 2008), and these studies also found that the same lineages will circulate in the same location for multiple years with low levels of genetic turnover. Phylogenetically characterizing the 2009 AIV from ducks from Mundy Pond would further contribute to our understanding of AIV dynamics in Newfoundland waterfowl.

2.4 Conclusion

This is the first multi-year study investigating AIV prevalence in seabirds, gulls and ducks in Newfoundland and Labrador. Viruses were identified in both Thick-billed and Common Murres. Due to the global distribution of AIV identifications in Common

and Thick-billed Murres, despite low surveillance effort, AIV is likely endemic in these two species. Both annual and seasonal patterns of prevalence likely played an important part in AIV detection in this study. The data suggest that the highest AIV prevalence in murres, particularly Thick-billed Murres in Newfoundland and Labrador is during the spring (February-June), however the total number of viruses is small. No viruses were isolated from any other seabird species, indicating that AIV infection is low or absent in these species during the summer months, at least for the years of this study. Viruses were isolated from Great Black-backed Gulls in Newfoundland in all years of the study, indicating that this species is an important AIV host in NL. No viruses were found in samples collected directly from American Herring Gulls, despite numerous isolations from this species in Delaware and New Jersey. Finally, although the characterized viruses from waterfowl were all from a single host species at a single location in a single year, there was sequence diversity. Therefore, AIV dynamics in NL waterfowl were similar to other locations in Canada and North America (Donis *et al.* 1989; Hatchette *et al.* 2004). However, this study nearly doubles the number of sequences available from AIV in American Black Ducks, and the first sequences from waterfowl in NL.

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Chapter 3

Reassortment of American and Eurasian genes in an influenza A virus (H13N2) isolated from a Great Black-backed Gull (*Larus marinus*), a species demonstrated to move between these regions

(Based upon a manuscript published in Archives of Virology with the same title)

3.1 Abstract

The primary hosts for influenza A viruses are waterfowl, although gulls and shorebirds are also important in global avian influenza dynamics. Avian influenza virus genes are separated phylogenetically into two geographic clades, American and Eurasian, which is caused by the geographic separation of the host species between these two regions. I surveyed a gregarious and cosmopolitan species, the Great Black-backed Gull (*Larus marinus*), in Newfoundland, Canada, for the presence of avian influenza viruses. I isolated and determined the complete genome sequence of an H13N2 virus, A/Great Black-backed Gull/Newfoundland/296/2008(H13N2), from one of these birds. Phylogenetic analyses revealed that this virus contained two genes in the American gull clade (PB1, HA), two genes in the American avian clade (PA, NA), and four genes in the Eurasian gull clade (PB2, NP, M, NS). I analyzed bird-band recovery information and found the first evidence of trans-Atlantic migration from Newfoundland to Europe (United Kingdom, Spain and Portugal) for this species. Thus, Great Black-backed Gulls could be important for movement of avian influenza viruses across the Atlantic Ocean and within North America.

3.2 Introduction

Wild birds are the primary reservoir for influenza A viruses, and typically carry low pathogenic strains (LPAIV) which can mutate and produce high pathogenic strains (HPAIV) (Webster *et al.* 1992). LPAIV have been isolated from at least 105 wild bird species across 26 different families (Olsen *et al.* 2006). The highest prevalence of infection occurs in waterfowl (Anseriformes) worldwide and shorebirds (Charadriiformes) in North America (Olsen *et al.* 2006). Influenza A also infects numerous mammalian species including humans, pigs, horses, mink, marine mammals, cats, and dogs (Webster *et al.* 1992; Kuiken *et al.* 2004; Crawford *et al.* 2005). Influenza A is a re-emerging disease in humans, causing yearly epidemics and also more irregular pandemics. The continual circulation of influenza A in host populations is maintained through the dynamic evolutionary capacities of the virus, with changes occurring through mutation leading to immune evasion (antigenic drift) and through genome segment reassortment after co-infection by more than one virus (antigenic shift) (Rambaut *et al.* 2008). These also contribute to the capacity of influenza A to move between host species (Webster *et al.* 1992; Salomon and Webster 2009).

Avian influenza A viruses (AIV) are broadly divided into two clades: American and Eurasian. This is due to limited overlap between North American and European bird migration flyways (Olsen *et al.* 2006). Exceptions occur in the Beringia region of Alaska and Russia (Ip *et al.* 2008; Koehler *et al.* 2008; Wahlgren *et al.* 2008), as numerous species, particularly waterfowl and shorebirds, move between Asia and Alaska each year (Winker *et al.* 2007). Due to the overlap of Old World and New World migratory pathways in Alaska, the likelihood of intercontinental reassortment events is

hypothesized to be greater in this area (Ito *et al.* 1995; Winker *et al.* 2007; Ip *et al.* 2008; Wahlgren *et al.* 2008). In contrast, the North Atlantic Ocean is a huge expanse of water between continents that limits bird movement. Therefore only highly adapted pelagic seabirds, including some gull species, able to live offshore indefinitely inhabit the North Atlantic. The prevalence of avian influenza is low in gulls (<0.1%-13%) (Olsen *et al.* 2006; Krauss *et al.* 2007; Munster *et al.* 2007; Velarde *et al.* 2010); however, a recent serological study indicated that large proportions of individuals in gull populations have been infected with AIV at some point in time (Velarde *et al.* 2010). With low prevalence of infection, it would seem less likely that reassortment events occur in these bird groups. However, genome sequences of AIV from Delaware Bay on the coastal Atlantic US indicate that intercontinental reassortment is occurring across the Atlantic Ocean (Makarova *et al.* 1999; Widjaja *et al.* 2004; Krauss *et al.* 2007; Munster *et al.* 2007; Dugan *et al.* 2008; Kishida *et al.* 2008).

Islands in eastern Newfoundland contain the most important concentration of breeding seabirds in eastern North America (Gaston and Jones 1998). This, in addition to open landfill sites and traditionally large fisheries, results in large breeding and wintering gull populations (Drury 1973; Drury 1974; Good 1998; Robertson *et al.* 2001). Species such as Great Black-backed Gull (*Larus marinus*) breed and have large over-wintering populations on the island of Newfoundland. Additionally, Arctic-breeding gull species and small numbers of various Eurasian gull species overwinter on Newfoundland. The timing of movements of these species is such that these American and Eurasian groups often overlap in insular Newfoundland during spring and fall, and there is potential for transmission of viruses from one group of gulls to another.

I surveyed wild birds for AIV in coastal Newfoundland and Labrador (NL), with emphasis on aquatic bird species such as seabirds and gulls that move large distances. I identified 2/38 Great Black-backed Gulls tested to be AIV positive between June 2008 and March 2009. The complete genome of one virus that could be cultured was sequenced, and I used phylogenetic analyses to show it contains several European gull-like segments. Finally, bird-band return information for this species was analyzed, which shows that this species moves across the North Atlantic, and therefore has the potential to move viruses between America and Eurasia.

3.3 Methods

3.3.1 Bird sampling

I tested 38 Great Black-backed Gulls in Newfoundland (Canada) for the presence of avian influenza from June 2008 - March 2009. I sampled 18 chicks in June and July 2008 on Gull Island (47°16'N, 52°46'W), 8 dead gulls in October of 2008 and 12 live captured birds (adult and immature) were captured between January and March 2009 in St. John's (47°34'52"N, 52°40'29"W). I swabbed the cloacal openings (life and dead birds) and the oropharynx (dead birds only) with a sterile-tipped applicator, which was then inserted into a tube containing Multitrans viral transport media (VTM) (Starplex Scientific, Etobicoke, Canada). Tubes containing samples were kept cool and placed at -80°C within 24 hours of collection.

3.3.2 Sample screening and virus culture

RNA was extracted from an aliquot of VTM from each swab sample using the MagMAX-96 Viral RNA Isolation Kit (Ambion, Streetsville, Canada) with an elution volume of 50 µL. Samples were assayed for the presence of the avian influenza matrix gene by real-time RT-PCR (rRT-PCR) (Spackman *et al.* 2002) using the QuantiTect Probe RT-PCR Kit (Qiagen, Mississauga, Canada) in a reaction volume of 20 µL.

Nine to 11 day old embryonated chick eggs (Charles River, North Franklin, Connecticut) were inoculated via the allantoic route to culture and isolate virus. Eggs were candled daily to monitor for embryo mortality. Two blind passages were performed, and allantoic fluid was tested for hemagglutinating activity after each passage.

3.3.3 RNA isolation and amplification of virus gene segments

Allantoic fluid was mixed with an equal volume of TriPure Isolation Reagent (Roche, Mississauga, Canada) and RNA was extracted using the MagMax AI/ND Viral RNA Isolation Kit (Ambion) following the manufacturer's instructions. cDNA was synthesized from the RNA sample using the Uni12M primer (Appendix 1) (Chan *et al.* 2006) and the Superscript III First Strand Synthesis System for Reverse Transcriptase PCR (Invitrogen, Burlington, Canada). Twenty-eight PCR reactions were then carried out to amplify the entire genome from this cDNA using a combination of primers (Appendix 1) (Zou 1999; Hoffmann *et al.* 2001; Phipps *et al.* 2004; Bragstad *et al.* 2005; Obenauer *et al.* 2006; Li *et al.* 2007; Koehler *et al.* 2008; Qiu *et al.* 2009). PCR products were purified using QIAquick PCR Purification Kit (Qiagen). Capillary sequencing of PCR products was carried out at The Centre for Applied Genomics (Toronto, Canada).

Complete segment sequences were assembled using Geneious v3.8.5 (Biomatters, New Zealand).

3.3.4 Phylogenetic analyses

The placement of the Great Black-backed Gull virus segment sequences within specific AIV lineages (American avian, Eurasian avian, American gull or Eurasian gull) was done through phylogenetic analyses that included representatives from these groups. Ten American avian and Eurasian avian reference sequences were selected from the NCBI Influenza Virus Resource Database (Bao *et al.* 2008). Representative virus sequences were selected from those isolated from duck species within the last 20 years. Sequences were selected from across North America including Alaska, Alberta, Minnesota and New York. Similarly, the selected Eurasian sequences were from viruses isolated in Russia, China and Japan. All available H13 and H16 gull viruses (as of November 2009) were also included (Appendix 2). Sequences were aligned using ClustalW v 1.4 and the resulting alignments used to construct neighbour-joining trees (Saitou and Nei 1987) with 10000 bootstrap replicates (Felsenstein 1985), all done within MEGA 4.0 (Tamura *et al.* 2007). To provide a more detailed view of the relationships between A/Great Black-backed Gull/Newfoundland/296/2008(H13N2) and other viruses, the subclades containing A/Great Black-backed Gull/Newfoundland/296/2008(H13N2) were subsequently reanalyzed using MrBayes v 3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) and 2,000,000 generations. Sequences from A/duck/Novosibirsk/02/05(H5N1), falling within the Eurasian avian clade, were used as outgroups for the PB2, PB1, PA, NP, M, and NS segments. The H16 and N3 gene

segments from A/shorebird/Delaware/168/06(H16N3) were used as outgroups for the HA and NA trees, respectively.

The H13 amino acid sequence alignment was constructed using ClustalX v 2.0 (Larkin *et al.* 2007). The Influenza Resource Database (Bao *et al.* 2008) alignment tool was employed to determine the amino acid positions.

The Eurasian gull clade of the matrix gene tree was used to construct a spatial tree with GeoPhyloBuilder v1.0.2. (Kidd and Liu 2008) and ArcGIS 9.3 (ESRI 2008). The resulting tree was projected using a polar orthographic projection.

3.3.5 Bird band returns

I analyzed data from the Bird Banding Office, Canadian Wildlife Service, Environment Canada for Newfoundland and Labrador Great Black-backed Gull banding (marked with individually coded rings placed on legs) and encounters (reports of banded birds). This comprised 3012 bird banding records and 69 encounter records from 1928 to July 2009. Bird-banding effort and encounters were categorized into the Avalon Peninsula (46°43'N-48°12'N, 52°46'W-54°13'W), central Newfoundland (47°54N-49°44'N, 54°14'W-57°33W), Burin Peninsula (46°46'N-47°54'N, 54°27W-56°01'W), western Newfoundland (47°29N-49°36'N, 57°34'W-58°27'W), the Northern Peninsula (49°37'N-51°37'N, 57°34W-58°4'W), and southern Labrador (51°29'N-52°53'N, 55°40'W-57°5'W). ArcGIS 9.3 (ESRI 2008) was used to build the map using a polar orthographic projection.

I also analyzed bird-band data for Great Black-backed Gulls banded in Europe and encountered in Greenland, Iceland and North America from EURING, the authority that maintains bird banding information in Europe (du Feu *et al.* 2009).

3.4 Results

3.4.1. AIV prevalence in Great Black-backed Gulls

In total, 38 samples were collected from Great Black-backed Gulls between June 2008 and March 2009 in NL, Canada. Two individuals sampled in October tested positive for avian influenza; all birds sampled in October (n=8) had died due to aspergillosis, a fungal infection caused by *Aspergillus fumigatus*. No viruses were detected in samples taken from chicks during the summer months on island breeding colonies (n=18) or from birds spending the winter in St. John's (n=12).

One virus was successfully cultured and subtyped as H13N2 by sequencing of the HA and NA segments [A/Great Black-backed Gull/Newfoundland /296/2008(H13N2)]. The second positive sample (A/Great Black-backed Gull/Newfoundland/355/2008) was a weak positive in the rRT-PCR assay, and presumably contained a very low titer. Attempts to culture virus from this weakly positive sample were unsuccessful and I was also unable to amplify any gene segments from the swab sample by conventional RT-PCR; therefore, no subtype or sequence information could be obtained. Sequences generated in this study have been deposited in GenBank under the accession numbers: GU724150-GU724157.

3.4.2. Relationship of A/Great Black-backed Gull/Newfoundland/296/2008(H13N2) to previously characterized viruses

Phylogenetic analyses revealed that the virus I isolated and characterized, A/Great Black-backed Gull/Newfoundland/296/2008(H13N2), comprised of segments with a mosaic pattern of relationships to previously characterized viruses from various avian influenza groups: Eurasian gulls (PB2, NP, M, NS), American gulls (PB1, HA), and American Avian (PA, NA) clades (Figure 3.1, Figure 3.2, Figure 3.3a, Appendix 2). The NP, M, and NS segments were most similar to A/shorebird/Delaware/168/06(H16N3) in addition to falling within the Eurasian gull clade (Figure 3.2) indicating that these Eurasian segments have been circulating within the North American system since at least 2006. Unlike the M, NS and NP segments, the PB2 segment of the Great Black-backed Gull virus was most similar to that of a virus isolated from a Laughing Gull (*Larus atricilla*) in New Jersey (CY042427), although it was also highly similar to A/shorebird/Delaware/168/06(H16N3), which fell in a sister group (Figure 3.2). These Eurasian PB2, M, NP, and NS segments have been identified most often in North American shorebirds, but have also been found in North American gulls (Figure 3.2). Although the PB2 segment fell within the Eurasian clade, this sequence appears well established within North America, having been isolated five times (Figure 3.2). The PB1 segment is most similar to shorebird and gull virus sequences forming a divergent clade from a larger American avian clade and a second American gull clade. The PA segment differed from A/shorebird/Delaware/168/06(H16N3) as it clustered in the American avian clade (Figure 3.1, Figure 3.2).

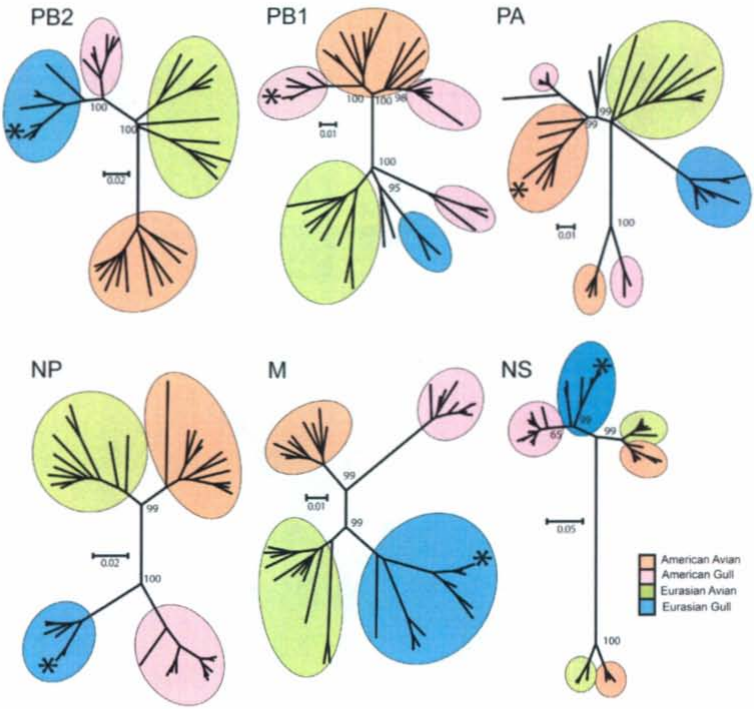

Figure 3.1 Phylogenetic analyses of A/Great Black-backed Gull/Newfoundland/296/2008(H13N2) PB2, PB1, PA, NP, M and NS segments. The trees are unrooted neighbour-joining trees of individual segments and contain representative viruses from the American avian, American gull, Eurasian avian and Eurasian gull groups, as indicated. Analyses for the PB2, PB1, PA, NP, M and NS genes are shown. A/Great Black-backed Gull/Newfoundland/296/2008(H13N2) is denoted by an asterisk (*). Scale bars indicate the number of nucleotide substitutions per site. GenBank accession numbers for all reference sequences are available in Appendix 2.

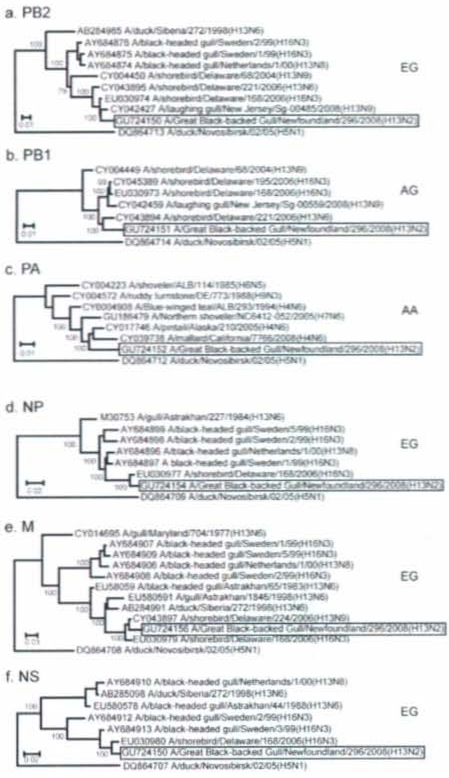

Figure 3.2 Bayesian analyses of the PB2, PB1, PA, NP, M and NS segments for the virus subgroup containing A/Great Black-backed Gull/Newfoundland//296/2008(H13N2) from Figure 1. The trees are rooted with A/duck/Novosibirsk/02/05(H5N1). a. Eurasian gull (EG) subclade of PB2 sequences, b. American gull (AG) subgroup of PB1 sequences, c. American avian (AA) subclade of PA sequences, d. Eurasian gull (EG) subclade of NP sequences, e. Eurasian gull (EG) subclade of M sequences, and f. Eurasian gull (EG) subclade of NS sequences. Bayesian posterior probabilities are indicated as percentages at major branch points. The scale bar indicates the number of nucleotide changes per site.

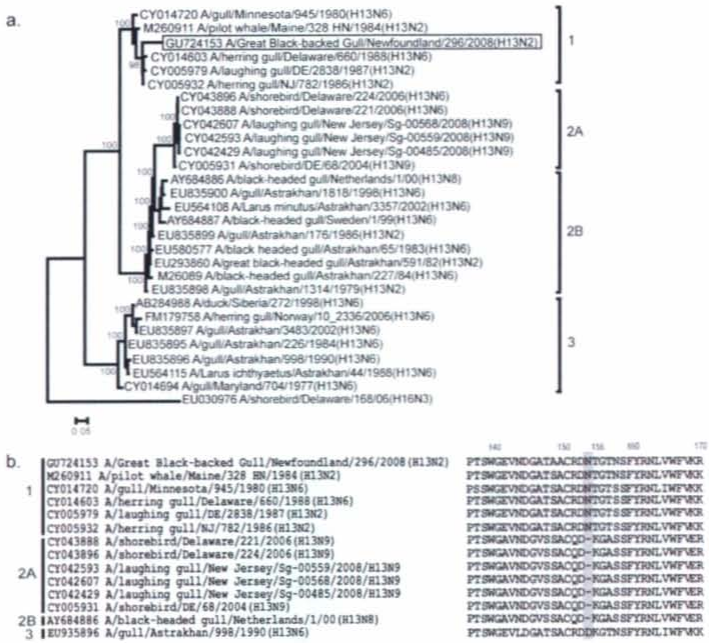

Figure 3.3 Analyses of H13 HA sequences. a. Phylogenetic analysis by Bayesian inference. The tree is rooted with A/influenza/shorebird/Delaware/168/06(H16N3). A/Great Black-backed Gull/Newfoundland/296/2008(H13N2) is indicated by a box in Clade 1, which also contains five H13 genes that circulated within the North American gull population in the 1980s. Clade 2A contains the H13 sequences most recently identified in the North American gull population. Bayesian posterior probabilities are indicated as percentages at major branch points. The scale bar indicates the observed number of nucleotide changes per site. b. A protein alignment of H13 genes, including representative sequences from each of the four clades indicated in a. The clade numbers are indicated on the left. An aspargine amino acid insertion is only present in clade 1, denoted by a shaded box. Single representative viruses from Eurasian origin clades 2B and 3 are included.

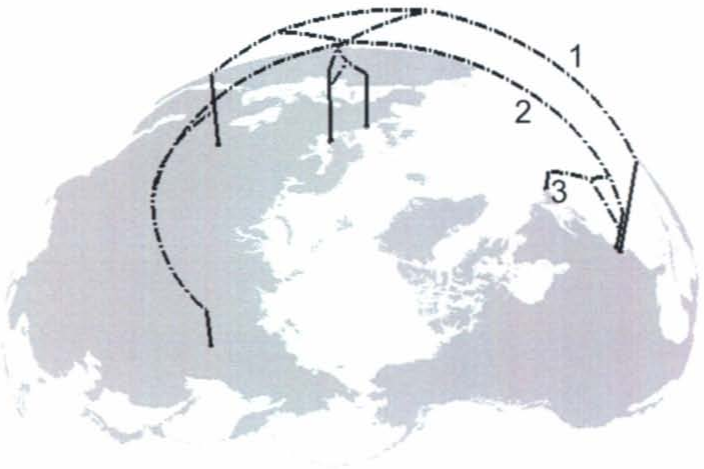

Figure 3.4 Spatial projection of the M segment phylogeny for the Eurasian gull clade. The phylogeny is presented in traditional form in Figure 3.2e, where all locations are described in the virus name. No outgroup sequence is included. The black circular points mark the individual virus isolation locations, the dotted lines represent the phylogenic tree backbone, and the complete lines represent the branches, where the length of each branch corresponds to the number of changes per site as determined by Bayesian analysis (Figure 3.2e). The numbers 1 and 2 indicate invasions of Eurasian genes into North America. The globe and phylogenetic information created in GeoPhyloBuilder is projected using a polar orthorgraphic projection, and the resultant three-dimensional globe is centered in the Arctic Ocean, north of the Chukchi Sea so that only North America and Eurasia are visible. Due to the curvature and position of the globe using this projection, Africa, South America, Australia and Antarctica are not visible. The island of Newfoundland is identified by the number 3.

A spatial projection of the matrix gene phylogeny for the Eurasian gull clade sequences (Figure 3.2) suggests there have been two separate invasions of this gene into North America (Figure 3.4). The virus corresponding to branch 1 (A/gull/Maryland/704/1977(H13N6)) was one introduction, but a similar gene has not been found in North America since that time. In contrast, branch 2 corresponds to three viruses: A/Great Black-backed Gull/Newfoundland/296/2008(H13N2), A/shorebird/Delaware/168/2006(H16N3) and A/shorebird/Delaware/224/2006(H13N9). Therefore this virus gene, presumed to be of Eurasian origin, is circulating within gulls and shorebirds in coastal Atlantic North America.

Unlike the segments discussed above, the HA gene was most similar to those from viruses identified in North America 20 years ago (Figure 3.3a, clade 1), rather than with sequences that have been found more recently (Figure 3.3a, clade 2A). The six viruses within clade 1 contain a 3-bp insertion corresponding to an inserted asparagine amino acid at position 154 that is unique to this clade of H13 sequences (Figure 3.3b). The NA gene was most similar to those circulating within North American ducks rather than American or Eurasian gulls (Appendix 5).

3.4.3. Great Black-backed Gull movement

Like most gull species found in eastern Canada, most Great Black-backed Gulls that breed in Newfoundland are thought to move south and spend the winter along the mid-Atlantic Coast (Good 1998). This notion is supported by an analysis of bird banding and encounter data in this region, which showed that birds banded in Newfoundland primarily migrate south to the northeastern United States (Figure 3.5). Aside from North

American returns, there are records of encounters for three different birds in western Europe since 2002: one in Spain, one in Great Britain and one bird encountered in twice in Portugal. These represent ~5% of all encounters of Great Black-backed Gulls banded in Newfoundland and Labrador. All three of these birds were banded as chicks on the Avalon Peninsula region of Newfoundland. Analysis of EURING records indicate that no Great Black-backed Gulls banded in Europe have been recorded in North America, but there is evidence that birds from the European population also move large distances. Three birds from the United Kingdom have been recovered in Iceland and birds from Iceland, Denmark, and western Russia have been recovered in Greenland (Lyngs 2003; du Feu *et al.* 2009). Great Black-backed Gulls are, apart from kittiwakes, the most commonly encountered gull species far from land in the western Atlantic (Brown *et al.* 1975).

3.5 Discussion

The AIV isolated from a Great Black-backed Gull in Newfoundland contains four segments of Eurasian viral descent. However, it is overall most closely related to another AIV isolated from coastal Atlantic North America, A/shorebird/Delaware/168/06(H16N3), with four segments of the two viruses (PB2, PB1, NP, M, and NS) showing close relationships. Therefore, these two viruses clearly share a recent common ancestor. However, there has also been reassortment that introduced

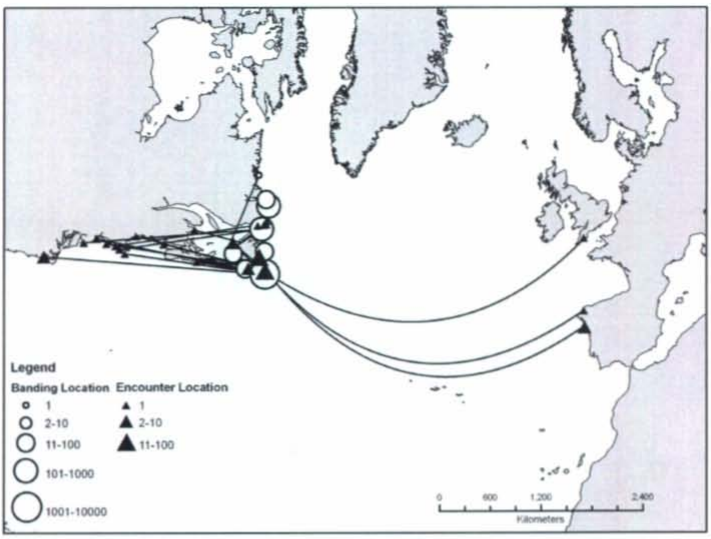

Figure 3.5 Records of movements of Great Black-backed Gulls banded in Newfoundland and Labrador, Canada. The circles show the banding effort of Great Black-backed Gulls in Newfoundland and Labrador, Canada from 1928 to July 2009. Triangles indicate worldwide encounter locations of Great Black-backed Gulls that were banded in Newfoundland and Labrador, and are connected to the location of banding by lines.

different PA, HA and NA segments between the viruses. Overall, the patterns I have observed indicate that some of these Eurasian segments have been circulating within North America for some time, and thus the Great Black-backed Gull virus is unlikely to represent a direct arrival from Eurasia.

Despite a high level of AIV surveillance effort in North America and Eurasia, a lack of targeted effort at gull populations, particularly in North America, is evident when analyzing the H13 phylogenetic tree. The virus from Newfoundland is the first found in 20 years in North America containing an H13 gene with a distinctive sequence insertion. It is likely that these genes have been circulating within North American gulls, but just that no viruses containing this unique gene have been detected.

Rather than following classical intracontinental migration patterns, numerous gull species have migration routes that include both intercontinental and intracontinental movements (Olsen and Larsson 2004). Most Great Black-backed Gulls from Newfoundland move along coastal Atlantic North America on an annual cycle banding data also demonstrate some trans-Atlantic movements. The subset of birds that move across the Atlantic are likely to comprise individuals that breed at the eastern edge of Newfoundland, and have only recently been detected due to an increase in bird banding effort in this area (Gaston *et al.* 2008). No such intercontinental movement of Great Black-backed Gulls has been recorded from populations in Maritime provinces of Canada (Gaston *et al.* 2008) or the United States (Gustafson and Hildenbrand 1999). Great Black-backed Gulls banded in Europe have not been found in North America, they do move large distances and have been recorded as far west as Greenland.

My analyses provide evidence that long-distance gull migration is contributing to movement of AIV genes between Eurasia and America. With the evidence that Great Black-backed Gulls are moving across the Atlantic Ocean, they may be playing an important role in moving viruses between these regions, as well as further circulating them within North American gulls and shorebirds during intracontinental movements. It is clear that AIV genome segments that appear to have originated in Eurasian gulls have moved to the North American system and are now circulating within that system. Increased surveillance in gulls for AIV, including in the northeastern United States and Canada, will be an important step towards better understanding the role of gulls in global AIV dynamics. This will also increase our understanding of intercontinental gene exchange and will aid in assessing the risk of invasion of potentially dangerous viruses and/or gene segments.

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Chapter 4

Extensive geographic mosaicism in avian influenza viruses from gulls in the northern hemisphere

(Based upon a manuscript submitted to PLoS Pathogens with the same title)

4.1 Abstract

Due to limited interaction of migratory birds between Eurasia and America, two independent avian influenza gene pools have evolved. There is evidence of low frequency reassortment between these regions, which has major implications for global AIV dynamics. Indeed, all currently circulating lineages of the PB1 and PA segments in North America are of Eurasian origin. Large-scale analyses of intercontinental reassortment have shown that viruses isolated from Charadriiformes (gulls, terns, shorebirds) are the major contributor of these reassortment events. To clarify the role of gulls in AIV dynamics, specifically in movement of genes between geographic regions, I sequenced six gull AIV genomes and analyzed these along with 135 other available virus sequences from gulls globally. Investigations of host species, spatial an temporal trends reveal biases in available sequence information. Despite these biases, phylogenetic analyses reveal a high frequency of geographic reassortment in gull viruses isolated in America. This intercontinental gene mixing is not found in viruses isolated from gulls in Eurasia. This study demonstrates that gulls are important as vectors for geographically reassorted viruses, particularly in America, and that more surveillance effort should be placed on this group of birds.

4.2 Introduction

Influenza A viruses, in the family *Orthomyxoviridae*, are enveloped and possess a genome consisting of eight unlinked segments of negative-sense single stranded RNA (Webster *et al.* 1992; Kawaoka *et al.* 2005). Wild birds are believed to be the primary reservoir for influenza A viruses, but they also have the capacity to infect a non-avian host species (Webster *et al.* 1992; Kuiken *et al.* 2004; Crawford *et al.* 2005). Influenza A viruses have dynamic evolutionary capabilities with genetic changes occurring through mutation and through genome segment reassortment after co-infection with two or more viruses (Webster *et al.* 1992; Rambaut *et al.* 2008; Salomon and Webster 2009). Most identified strains of avian influenza A viruses (AIV) are low pathogenic (LPAIV), which are carried without readily apparent clinical signs. Highly pathogenic (HPAIV) strains can cause significant morbitity and mortality in both wild birds and poultry (Alexander 2007). LPAIV viruses have been isolated from at least 105 wild bird species in 26 different families, although the highest prevalence of infection occurs in Anseriformes (ducks, geese, swans) and Charadriiformes (shorebirds, gulls and terns) (Olsen *et al.* 2006).

AIV are broadly divided into two clades, American and Eurasian (Webster *et al.* 1992; Olsen *et al.* 2006), believed to be a result of limited overlap in American and Eurasian bird ranges (Olsen *et al.* 2006). However, movement of viruses between these regions occurs because viruses with American sequences are found in Eurasia, and vice-versa (Krauss *et al.* 2007; Dugan *et al.* 2008; Chen and Holmes 2009). Unlike waterfowl, many gulls undergo intercontinental, pelagic and intracontinental movements (Olsen and Larsson 2004). AIV sequences from gulls frequently form different clades from those

isolated from other wild bird hosts (Kawaoka *et al.* 1988; Obenauer *et al.* 2006; Dugan *et al.* 2008; Hanson *et al.* 2008), including the hemagglutinin subtypes H13 and H16 that have been characterized as gull-specific (Hinshaw *et al.* 1982; Yamnikova *et al.* 2003; Fouchier *et al.* 2005). Large-scale analyses of AIV genome sequences have demonstrated a low frequency of intercontinental reassortment events across all wild bird hosts, and it has been suggested that all H13 and H16 viruses contain a genome with a mosaic of geographic origins (Krauss *et al.* 2007; Dugan *et al.* 2008).

Intercontinental movements of birds and virus segments have large implications for AIV population structure. It has been demonstrated that Eurasian segments have invaded and displaced currently circulating American lineages; indeed, all currently circulating PB1, PA, and H6 lineages in North America are of Eurasian origin (Bahl *et al.* 2009; zu Donha *et al.* 2009). To clarify the role that gulls play in global AIV dynamics I sequenced the complete genomes of six AIV from gulls in Alaska, greatly increasing the genomic information available for that host group from that region of North America. Alaska has been identified as an important area for mixing of numerous bird species from Eurasia and North America (Winker and Gibson 2010). The analyses of these new AIV sequences integrated with all other available AIV sequences from gulls will allow us to evaluate historical gull surveillance globally, the phylogeography of gull AIV, gull specific lineages on both a local and global scale, and the frequency of intercontinental reassortment of AIV gene pools. My analyses reveal a remarkably high frequency of geographic reassortants in gull AIV isolated in America, particularly among the six viruses characterized in this study. Seven of eight segments from five of these AIV were more similar overall to sequences from viruses detected in Eurasia than in North

America, indicating that Alaska is important in intercontinental movement in context of both waterfowl and gull AIV. Not all H13 and H16 AIV are reassorted, as intercontinental gene mixing is not observed in AIV isolated from Eurasian gulls. Further, intercontinental exchange is not limited to the H13 and H16 viruses isolated in North America, but is also found in viruses with other HA subtypes detected in gulls. This study demonstrates that gulls are important for geographic reassortment of AIV, and are likely one of the major host groups involved in the movement of AIV genes from Eurasia to America.

4.3 Materials and Methods

4.3.1 Virus isolation and characterization

Fecal samples were collected from Glaucous-winged Gull (*Larus glaucascens*) roosting sites at the city dock (60.547N, -145.785W), Hartney Bay (60.499N, -145.867W) and Odiak Slough (60.539N, -145.786W) in Cordova, Alaska. Individual, freshly deposited feces from roosting locations used exclusively by gulls were swabbed using a sterile-tipped applicator, which was then inserted into a tube containing M4RT viral transport media (VTM) (Remel, Lenexa, KS).

Screening for influenza A was performed using a two-step real-time RT-PCR approach (Runstadler *et al.* 2007). Briefly, RNA was extracted from each swab sample using the MagMAX-96 Viral RNA Isolation Kit (Ambion, Austin, Texas) following the manufacturer's instructions. cDNA was synthesized using the M-MLV reverse transcriptase enzyme (Invitrogen, Carlsbad, California) and random hexamers (Invitrogen) and assayed for the presence of the AIV matrix gene by real-time PCR

(Spackman *et al.* 2002) using a TaqMan (Qiagen, Valencia, California) assay with a threshold cut-off (Ct) < 40. Virus isolation was carried out in 9 to 11 day old SPF embryonated chicken eggs (Charles River, North Franklin, Connecticut) inoculated via the allantoic route. The eggs were candled daily, for up to 7 days, to monitor for embryo mortality. Up to three blind passages were performed, and allantoic fluid was assayed for the presence of the avian influenza matrix gene after each passage as previously described (Runstadler *et al.* 2007).

For virus genome sequencing, cDNA was synthesized using the Uni12M primer (Chan *et al.* 2006) and the Superscript III First Strand Synthesis System for Reverse Transcriptase PCR (Invitrogen) with RNA extracted from the allantoic fluid. Twenty-eight PCR reactions were then carried out in order to amplify the entire genome using a combination of primers (Appendix 1) (Zou 1999; Hoffmann *et al.* 2001; Liu *et al.* 2004; Phipps *et al.* 2004; Bragstad *et al.* 2005; Chan *et al.* 2006; Obenauer *et al.* 2006; Koehler *et al.* 2008; Qiu *et al.* 2009). PCR products were purified using the QIAquick PCR Purification Kit (Qiagen). Capillary sequencing of PCR products was carried out at The Centre for Applied Genomics (Toronto, Canada). Complete segment sequences were assembled using Geneious v3.8.5 (Biomatters, New Zealand). Sequences generated in this study have been deposited in the NCBI GenBank database and the Influenza Resource Database under the accession numbers CY070847 – CY070894.

4.3.2 Sequence analyses

For the analyses presented here, I incorporated the full genome sequences I completed for six gull isolates from Alaska with sequences available at the National Centre for Biotechnology Information (NCBI) GenBank and Influenza Resource

Databases (Bao *et al.* 2008). All sequence data available from AIV isolated from gulls (as of April 2010) were retrieved (Appendix 6). Inconsistencies in virus names were resolved by checking associated publications when available. Viruses with more than one sequence per segment were further investigated, and sequences were compared using BLAST (Altschul *et al.* 1997); the most complete or most recent sequences were selected. Suspected language translation errors in host species names in GenBank were investigated to determine the correct host species based upon range distributions (Gillmor *et al.* 1998) and by translation of original foreign literature. Partial and complete sequences were considered when analyzing geographic assignment of segments and basic subtype diversity, but only complete segment sequences (within 50 base pairs of each end) were included in the construction of phylogenetic trees. A total of twenty non-gull virus sequences were included to represent the American and Eurasian avian clades, and were selected from various host species, locations, years and subtypes (Appendix 2). The H13 and H16 phylogenetic trees were constructed using all available H13 and H16 sequences, including those isolated from birds other than gulls (Appendix 7).

Complete sequences were aligned using ClustalW version 1.4 and the resulting alignments used to construct neighbour-joining trees (Saitou and Nei 1987) with 10000 bootstrap replicates (Felsenstein 1985), all done within MEGA 4.1 (Tamura *et al.* 2007). Geographic reassortment was determined by both phylogenetic and BLAST analyses. A geographic reassortant was defined as a sequence from a virus isolated in one geographic region that was similar to a well established clade of viruses detected in a different geographic region.

4.4 Results and Discussion

4.4.1 Host, spatial and temporal trends in historical gull AIV identification

In addition to the six viruses sequenced in this study, partial and/or complete sequence information was available from 135 other AIV isolated from gulls globally between 1975 and 2009. Many other viruses have been detected in or isolated from gulls, but they have not been sequenced (Olsen *et al.* 2006; Krauss *et al.* 2007; Munster *et al.* 2007; Ip *et al.* 2008; Parmley *et al.* 2009; Pasick *et al.* 2010; Velarde *et al.* 2010). Future characterization of these additional viruses would be beneficial for better tracing gull virus evolution through space and time.

The AIV analyzed in this study were isolated from numerous different gull species with different migratory patterns, feeding behaviours, and life history strategies, and which are therefore representative of the diverse spectrum within the family Laridae, even if full geographic representation is not present (Pons *et al.* 2005) (Table 4.1). There are biases towards certain species, such as Black-headed Gull (*Chroicocephalus ridibundus*) and Great Black-headed Gulls (*Larus ichthyaetus*) that have been targeted in the Volga Delta of the Russian Federation (Roslaya *et al.* 1984; Iamnikova *et al.* 2009), and American Herring Gulls (*L. smithsonianus*) and Laughing Gulls (*L. atricilla*) that have been targeted in the Atlantic coast of the United States (Table 4.1). Most sequenced viruses from North America are from the eastern coast (55 of 66), and only 8 viruses isolated in Alaska, including the 6 sequenced in this study, represent the Pacific coast. Viruses have been sequenced from more locations in Eurasia, but few coastal regions of Eurasia are represented (Figure 4.1). The migratory patterns of gulls dictate that if geographical reassortments of AIV were occurring in Alaska or eastern North America,

the Eurasian segments would most likely originate in coastal regions including Kamchatka, Japan, China, Greenland, Iceland, Spain, Portugal, and the United Kingdom (Olsen and Larsson 2004); unfortunately, there are currently no LPAIV sequences available from gulls from these regions of Eurasia. A single gull AIV from South America has been sequenced, and there are no gull viruses available from Australia or Africa (Figure 4.1). South Africa is significant in the history of AIV surveillance in wild birds, with the first documented case of HPAIV in wild birds occurring in terns, a sister group of gulls, there in 1961 (Becker 1966)

The availability of AIV sequences also is not uniform along a temporal scale, and virus isolations are clustered within specific time periods (Table 4.2). The majority of gull viruses sequenced in the Americas were isolated between 1985-1989 and after 2000, while only 2 partial genome sequences are available from viruses identified during the 1990s. In Eurasia, there has been steady isolation and sequencing of viruses from the Volga River Delta, Russian Federation (Yamnikova *et al.* 1989; Yamnikova *et al.* 1989).The number of virus sequences has also increased since 2000, partially due to mass mortality events associated with H5N1 outbreaks in China (Wang *et al.* 2008; Kou *et al.* 2009). Work in Eurasia in 1999 and 2000 led to the description of a new subtype, H16, in European Black-headed Gulls (Fouchier *et al.* 2005).

4.4.2 Subtype diversity and distributions

The distribution of AIV sequences between America and Eurasia is fairly even (Table 4.2). Of the 142 AIV with sequence data available, many only have sequence information available for the HA segment, particularly amongst the Eurasian viruses.

Table 4.1: Species in the family Laridae from which AIV sequence data are available.

Species	Number of viruses
American Herring Gull (*Larus smithsonianus*)	15
Black-headed Gull (*Chroicocephalus ridibundus*)	16
Black-legged Kittiwake (*Rissa tridactyla*)	1
Brown-headed Gull (*Chroicocephalus brunnicephalus*)	6
Common Gull (*Larus canus*)	2
Glaucous Gull (*Larus hyperboreus*)	1
Glaucous-winged Gull (*Larus glaucescens*) [this study]	6
Great Black-backed Gull (*Larus marinus*)	1
Great Black-headed Gull (*Larus ichthyeatus*)	18
Herring Gull (*Larus argentatus*)	9
Kelp Gull (*Larus dominicanus*)	1
Laughing Gull (*Larus atricilla*)	22
Little Gull (*Larus minutus*)	1
Mediterranean Gull (*Larus melanocephalus*)	1
Ring-billed Gull (*Larus delawarenis*)	5
Sabine's Gull (*Larus sabini*)	1
Slaty-backed Gull (*Larus schistisagus*)	1
Slender-billed Gull (*Chroicocephalus genei*)	1
Yellow-legged Gull (*Larus michahellis*)	1
Vega Gull (*Larus vegae mongolicus*)	2
Unknown gull species	31

Common and latin names follow the IOC guidelines.

Figure 4.1: Locations of gull AIV identifications for which sequence data are available, 1975-2009. There is only a single virus isolated from the southern hemisphere, isolated from a bird in Patagonia. Few viruses have been characterized from birds tested in coastal regions of Europe or Asia. There are numerous locations from which viruses have been sequenced in eastern North America, and only a single along the Pacific coast of North America.

Table 4.2: Availability of gull AIV sequence data by virus identification date.

Year	Region	
	America	Eurasia
1975-1979	7	7
1980-1984	5	8
1985-1989	23	6
1990-1994	1	2
1995-1999	1	7
2000-2004	12	15
2005-2009	17	31
Total (142)	66	76

Approximately half have sequence data available for the segments other than HA and NA: PB2 (n=64), PB1 (n=67), PA (n=66), NP (n=75), M (n=78) and NS (n=71) (Appendix 8), and 51 viruses have complete genome sequences available. Nearly half of the Eurasian sequences are HPAIV H5N1 viruses (Appendix 8).

All HA subtypes are represented in AIV from gulls, with the exceptions of H8 and H15. Viruses from Eurasia and America have different HA subtype trends, with the Eurasian viruses dominated by H5, H13 and H16, whereas the American gull viruses have higher subtype diversity and more subtype H2, H6 and H7 viruses (Figure 4.2A). The H13 and H16 subtypes are adapted for recognition of fucosylated sialyloligosaccharide receptors, which is required for virus attachment and hence efficient replication in gull intestinal cells (Yamnikova *et al.* 2003; Matrosovich *et al.* 2008). It has also been demonstrated that American gull H4 viruses are adapted for recognition to these receptors (Matrosovich *et al.* 2008), increasing the fitness of these viruses in gulls. All NA subtypes have been found in gull viruses in America, while N7 and N9 have not been detected in Eurasia (Figure 4.2B). Overall, the most common subtype combinations identified in gulls (excluding H5N1) are H13N6, H16N3, H13N2 and H13N9, whereas most other subtype combinations have only been identified 3 or fewer times (Appendix 9). There are apparent linkages between H13 and N6, and H16 and N3, indicating that there is increased fitness in gulls for these subtype combinations.

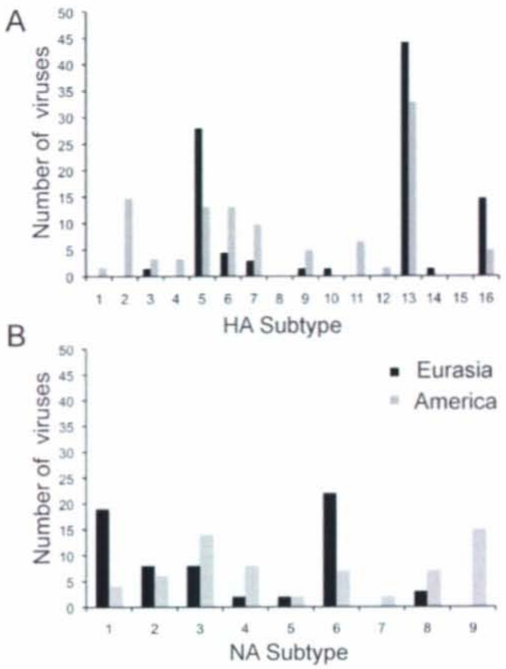

Figure 4.2: Subtype diversity within AIV from gulls in America and Eurasia. A is the distribution of hemagglutinin types. B is the distribution of neuraminidase subtypes.

4.4.3 Phylogeography of gull AIV

My phylogenetic analyses with individual segment sequences have provided new insights into AIV dynamics in gulls. As with other AIV, there is a clear phylogeographic pattern for gull AIV, with most viruses isolated in America clustering together and viruses isolated in Eurasia clustering together (Figure 4.3). It has previously been demonstrated that there are gull-specific clades (Olsen *et al.* 2006); however, some gull virus sequences are also integrated amongst viruses isolated from other host groups (Figure 4.3). Gull-specific lineages of the M segment are more similar to avian clades of the same geographic origin, with the exception of one American gull clade that is most similar to Eurasian reference sequences (Figure 4.3, group 1). To the contrary, the Eurasian and American gull-specific clades of the NP and NS trees are more similar to each other than they are to the avian clades from the same geographic region. Gull-specific clades for these segments suggest that these forms have increased fitness in gulls, which therefore may have implications for host-range restrictions. These American gull-specific clades are dominated by AIV from the 1980s (Figure 4.3, Table 4.3), whereas the viruses isolated more recently are found in both gull-specific lineages and those containing waterfowl reference sequences. BLAST analyses of the sequences in these gull-specific clades verify the absence of a close relationship to any waterfowl viruses. However, viruses from shorebirds, particularly Ruddy Turnstones (*Arenaria interpres*), are highly similar to the gull-specific groups 1 (up to 15 shorebird viruses) and 2 (up to 9 shorebird viruses) (Figure 4.3). The polymerase-encoding segments (PB1, PB2 and PA) do not have clearly defined gull clades, but rather the gull viruses are generally integrated with other AIV sequences. All but two of the 71 gull viruses analyzed, A/laughing

gull/NY/2455/00(H7N3) and A/gull/Astrakhan/1846/1998(H13N6), contain the same NS subtype (subtype A; Figure 4.3).

Most North American gull AIV sequences are from Delaware Bay, mainly from the years 1986-1989. My analyses show that there were two different virus lineages circulating in the gulls there at that time. One lineage comprised mostly H2 viruses (Figure 4.3, group 1; Table 4.3), and the other H13 viruses (Figure 4.3, group 2; Table 4.3). These two groups of viruses are distinct in five of the six trees (PB2, PA, NP, M, and NS). Two viruses are reassorted between these two groups, A/herring gull/Delaware/471/86(H2N7) and A/laughing gull/NJ/798/1986(H2N7), but the full extent of reassortment is unclear because the full genome sequences are not available (Appendix 6). Although there are lower numbers of AIV sequences from subsequent years, it appears that some descendant segments of each lineage still persist.

My analyses also indicate that the PB2, PB1, PA, NP, M, NS are somehow linked to the HA segment. This is based on the findings that the H13 segments of the group 2 viruses (Table 4.3) are similar (Figure 4.4) and the H2 segments of the group 1 viruses (Table 4.3) are similar (Appendix 10). Also, the H2 viruses in group 1 (Table 4.3) are more similar to Eurasian viruses than they are to American avian or other American gull H2 viruses (Appendix 10). It has been proposed that the genetic structure of AIV is in part a result of transient genetic linkage between PB2, PB1, PA, NP, M, segments and the HA and NA, and perhaps NS, segments (Chen and Holmes 2010). Therefore, it is possible that linkage between the H2 and M segments in the group 1 viruses resulted in the introduction of Eurasian M segments into North America. The PB2 segment of the group 2 viruses and the M segments of the group 1 viruses are more similar to Eurasian

viruses than they are to American avian or gull viruses, which demonstrates the introduction and circulation of these Eurasian genes in Delaware Bay gulls (Figure 4.3; Table 4.3).

Similar to the two different American clades of gull AIV found in Delaware Bay in the 1980s, there are two very different H13 HA lineages circulating in the gull population of the Caspian Sea, Russian Federation (Yamnikova *et al.* 1989). The Eurasian clade 1 (Figure 4.4) is more similar to two clades of American viruses: one lineage that was circulating during the 1980s and one that contains viruses currently circulating in North America. The Eurasian clade 2 (Figure 4.4) is similar to two American viruses from the 1970s, as well as the five H13 viruses that were isolated in Alaska and sequenced in this study. Eurasian viruses dominate the H16 portion of the tree, with the exception of a single North American lineage that includes viruses from 1975, 1986, 1988, and the Alaskan H16 virus isolated in this study. Two H16 sequences from shorebirds in Delaware Bay in 2006 are Eurasian in origin and are most similar to Eurasian clade 3. The combination of recent evolution (Chen and Holmes 2010), recent discovery (Fouchier *et al.* 2005), and few H16 HA sequences, makes characterizing the dynamics of these viruses very challenging.

Analyses of the most common NA subtypes, N3 and N6, reveal that these sequences are largely separated into distinct North American and Eurasian lineages, but the presence of Eurasian sequences in the American viruses is evident from both the N3 and N6 trees (Figure 4.4). Indeed, all NA segments from the Alaskan viruses sequenced

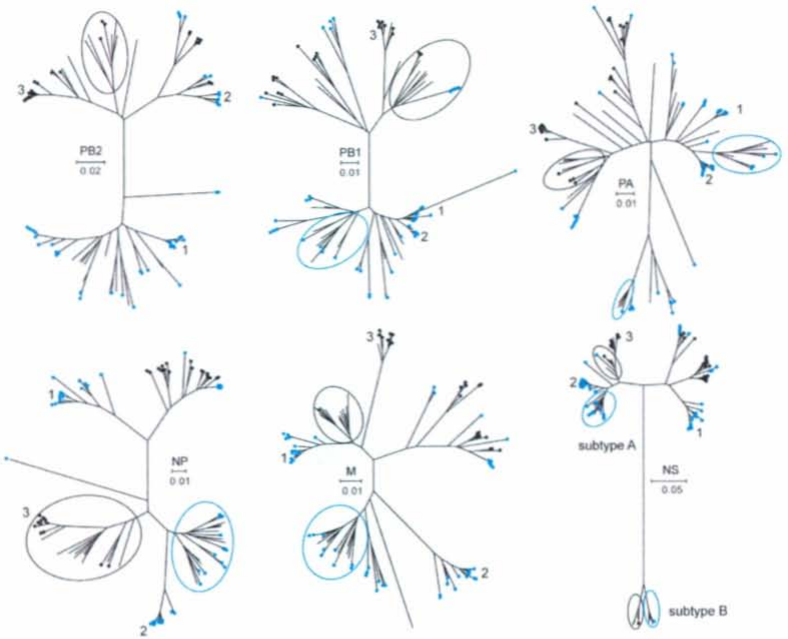

Figure 4.3: Neighbour-joining trees of PB2, PB1, PA, NP, M, and NS segments. Viruses isolated from gulls are denoted by dots at the ends of branches, with those isolated in America indicated in blue and those isolated in Eurasia indicated in black. Reference AIV sequences from other avian taxa do not have points at the ends of branches. Circles indicate where the reference sequences cluster together by geographic origin, with clusters of viruses isolated in America indicated in blue and clusters of viruses isolated in Eurasia indicated in black. Where no circles are present, reference sequences do not cluster into a well-defined clade. The clades denoted 1 and 2 on each panel consist of viruses that consistently group together (identified in Table 4.3). The clade marked with a 3 on each panel represents HPAIV H5N1 viruses. The NS subtypes, A and B, are indicated on the NS tree. Scale bars indicate the number of substitutions per site. Reference sequences are identified in Appendix 2 and All gull AIV sequences are identified in the Appendix 6.

Table 4.3: Identification information for AIV from the Delaware Bay gull community (1986-1989), comprises two distinct clades based on phylogenetic analyses of the PB2, PB1, PA, NP, M, and NS segments.

Name	Subtype	Year	Clade
A/laughing gull/DE/2718/1987(H9N5)	H9N5	1987	1
A/herring gull/DE/698/1988(H2N1)	H2N1	1988	1
A/herring gull/DE/677/1988(H2N8)	H2N8	1988	1
A/herring gull/DE/692/1988(H2N8)	H2N8	1988	1
A/herring gull/DE/703/1988(H2N8)	H2N8	1988	1
A/herring gull/DE/670/1988(H2N9)	H2N9	1988	1
A/herring gull/NJ/402/1989(H5N3)	H5N3	1989	1
A/herring gull/NJ/406/1989(H5N3)	H5N3	1989	1
A/laughing gull/NJ/276/1989(H6N8)	H6N8	1989	1
A/herring gull/NewJersey/780/86(H1N3)	H1N3	1986	2
A/herring gull/DE/475/1986(H13N2)	H13N2	1986	2
A/herring gull/NJ/782/1986(H13N2)	H13N2	1986	2
A/laughing gull/DE/2838/1987(H13N2)	H13N2	1987	2
A/herring gull/DE/665/1988(H4N6)	H4N6	1988	2
A/laughing gull/DE/554/1988(H13N3)	H13N3	1988	2
A/herring gull/DE/660/1988(H13N6)	H13N6	1988	2
A/herring gull/DE/712/1988(H16N3)	H16N3	1988	2

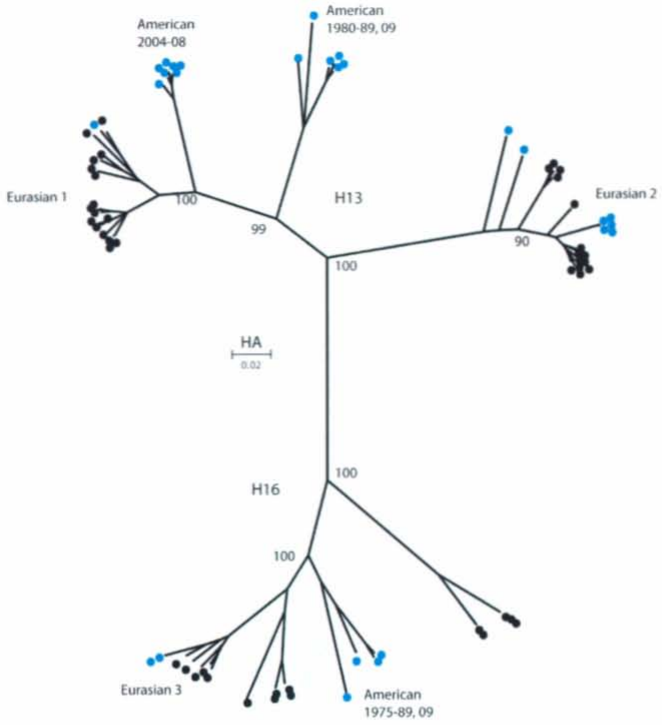

Figure 4.4: Neighbour-joining tree of all H13 and H16 complete sequences. Viruses isolated in Eurasia are indicated with black dots and those isolated in America are indicated with blue dots. The scale bar indicates the number of substitutions per site. Bootstrap values are provided as percentages based on 10000 replicates for selected major branch points. The identification information for the sequences is provided in Appendix 7.

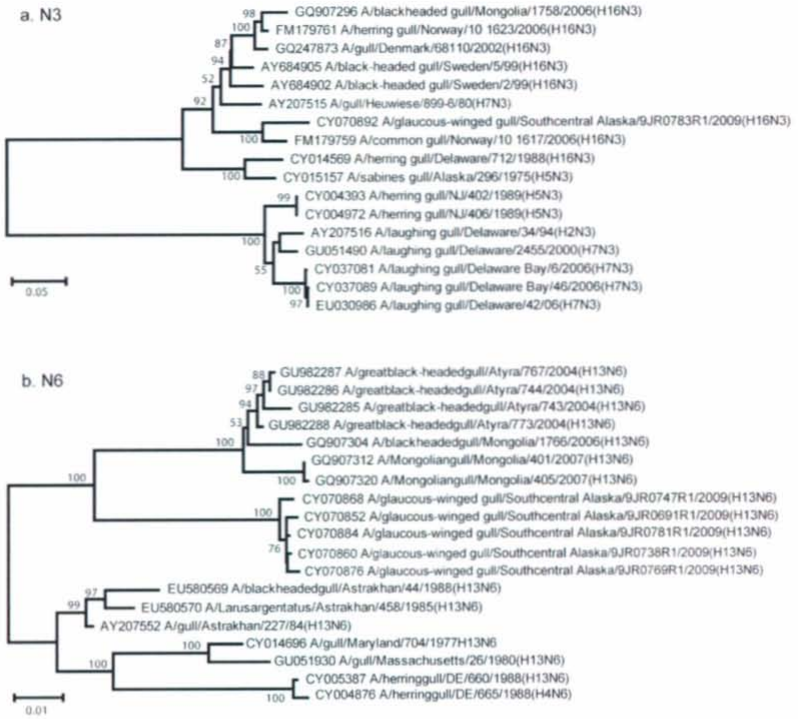

Figure 4.5: Neighbour-joining trees of the most common NA subtypes of gull viruses. A contains the available complete N3 sequences and B contains the available complete N6 sequences from gull viruses. The scale bar indicates the number of substitutions per site. Bootstrap values are provided as percentages based upon 10000 replicates.

in this study are more similar to sequences from Eurasian viruses than those from other gull viruses in America. Furthermore, the N6 sequences from the Alaskan gulls are also distinct from the most closely related Eurasian lineage (Figure 4.4), suggesting poor sequence coverage of viruses from gulls and/or the existence of unique lineages of viruses in western North America. The N6 tree also indicates there are two different Eurasian groups (Figure 4.4).

4.4.4 Pervasive inter-regional reassortment in gull AIV

It was observed previously that geographical reassortment between Eurasia and America is much higher in gull-specific H13 and H16 lineages than in other AIV subtypes (Dugan *et al.* 2008). My study indicates that, not only is reassortment prevalent in American H13 and H16 viruses, but also in other subtypes isolated from gulls (Figure 4.6). To the contrary, there is no evidence of reassortment in Eurasian gull viruses (Figure 4.3, Figure 4.4). A single Eurasian gull virus with an American segment has been identified, where the HA segment of an H9N2 virus isolated in southern France is of American origin (Lebarbenchon *et al.* 2009). There are few cases of geographic reassortant virus discovery in Eurasia overall, with the exceptions including viruses from a pelagic seabird, Common Murre (Wallensten *et al.* 2005), and individual cases in Italy (Fusaro *et al.* 2009), India (Pawar *et al.* 2010), and Japan (Liu *et al.* 2004). As previously mentioned, the apparently low levels of intercontinental reassortment in Eurasia may be an artifact resulting from a lack of AIV sequences from specific coastal regions where mixing of American and Eurasian species, especially gulls, may be occurring.

Most gull viruses found in America contain segments with a mosaic phylogeographic pattern (Figure 4.6). Thirty-five of the 63 American viruses have at least one segment that falls within Eurasian lineages in phylogenetic analyses. Of the 27 viruses that were not reassorted, 19 genomes (PB2, PB1, PA, NP, M, NS) were incomplete. Most notably, only one H13 virus for which a complete genome sequence is available, A/kelp gull/Argentina/LDC4/2006(H13N9), is not geographically reassorted. However, numerous gull AIV with HA subtypes more frequently found in ducks and shorebirds are also geographically reassorted. Most of the identified geographically reassorted segments appear to have resulted in successful invasion of North America because few of these sequences are similar to only Eurasian viruses; rather, most of these Eurasian sequences have been detected in North America more than once (Figure 4.6). Indeed, it is evident that some of these Eurasian lineages have diversified and are well established in North America, as is demonstrated for the M segment (Figure 4.3, group 1).

The Alaskan viruses sequenced in this study contain the highest numbers of Eurasian segments ever found in viruses in North America. The five H13 viruses each had seven Eurasian segments and the H16 virus had six. No AIV has been detected in North America with all eight segments of Eurasian origin, but my isolates suggest it might be possible that such a virus will be found in Alaskan gulls.

Figure 4.6: Geographical reassortment in AIV isolated from gulls in America. Each segment is represented by a box, and ordered by segment size from left to right (PB2, PB1, PA, NP, M, and NS). Light grey boxes indicate clustering within an established clade of North American viruses, dark grey indicate that a lineage of two or more American viruses that are geographically reassorted and black boxes indicate clustering of a single American viruses within a clade of Eurasian viruses. White boxes indicate no sequence information is available. Segments with partial sequence information available are included. The viruses are ordered by year subtype.

4.5 Concluding Remarks

Understanding of the pattern of AIV dynamics in gulls is still somewhat limited due to the poor uniformity of sequence availability over spatial, temporal and host species ranges. A more complete dataset would enable a better description of the introduction and extinction of lineages in this host group, and specifically concerning reassortment events that are of great interest in AIV dynamics. Particularly, viruses from the Southern Hemisphere are greatly lacking from the current data.

The origin of Eurasian segments in North America has long been questioned, and previous studies have investigated ducks and shorebirds as potential vectors of these viruses (Krauss *et al.* 2007; Pearce *et al.* 2009). Although gulls have been recognized to carry AIV with unique subtypes and geographic reassortants (Makarova *et al.* 1999; Macken *et al.* 2006), this is the first independent analysis of all currently available gull virus sequences. Although Eurasian gulls do not seem to have AIV that contain American segments, analyses of the spatial, temporal and host species origin of the available sequences indicate poor coverage overall, especially in important coastal regions. My analyses suggest that gulls are important mixing vessels for viruses and are possibly the main contributors of geographically reassorted viruses in North America. Furthermore, Alaska is indeed an important location to survey for Eurasian viruses, but more emphasis needs to be placed upon gulls in this location, and others.

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Chapter 5

Conclusion

5.1 Summary

Newfoundland is home to the most important concentration of breeding seabirds in eastern North America (Gaston and Jones 1998). The co-occurrence of numerous species from broad geographic ranges provides a unique opportunity to potentially detect a diverse array of avian influenza viruses (AIV). For wild birds, studies on AIV in waterfowl have completely overshadowed those on any other host groups by number, variety and distribution (Alexander 2007). The goal of my study was to investigate AIV infection in under-studied host groups that are abundant in Newfoundland and Labrador (NL), such as seabirds and gulls, in addition to the local waterfowl community.

Currently described AIV, predominately isolated from waterfowl, are broadly divided into two main clades, American and Eurasian (Olsen *et al.* 2006), with a low frequency of intercontinental movement of virus sequences between these regions (Krauss *et al.* 2007; Bahl *et al.* 2009; Chen and Holmes 2009). Intercontinental gene exchange has large implications in the influenza gene pool; indeed, all known PB1, PA, and H6 virus segments currently circulating in North America are of Eurasian origin (Bahl *et al.* 2009; zu Donha *et al.* 2009; Chen and Holmes 2010). Unlike waterfowl, many gulls and seabirds have pelagic and intercontinental ranges, and due to this life history strategy these species could be important hosts of AIV in terms of global virus dynamics, regardless of low prevalence. Furthermore, gulls from Newfoundland move

along the Atlantic coast (Gaston *et al.* 2008), and therefore could be an important vector for the movement of Eurasian segments in and into North America.

For seabirds, AIV have previously been isolated only from shearwaters in Australia (Downie *et al.* 1973; Downie *et al.* 1977), Thick-billed Murres (*Uria lomvia*) in Alaska (Ip *et al.* 2008), and Common Murres (*U. aalgae*) in Alaska (Ip *et al.* 2008), Sweden (Wallensten *et al.* 2005) and Russia (Sazonov *et al.* 1977). However, additional species have been surveyed in small numbers (as reviewed by Olsen *et al.* 2006). A single complete (Wallensten *et al.* 2005) and two partial (Obenauer *et al.* 2006) genome sequences are available from Common Murres. In this work, Common Murre and Thick-billed Murre were the only seabird species from which AIV were detected. Furthermore, viruses were detected in Thick-billed Murres in NL in the winter and spring (Feb-April), suggesting a seasonal effect. Previous AIV surveillance in seabird species has occurred during the summer months, and therefore low reported prevalence could be a result of ineffective sampling strategies. The genome sequence of one of these viruses, A/Thick-billed Murre/Newfoundland/031/2007(H11N2), was unlike other available sequences from murres. It did not have segments from both Eurasian and American origins, but rather was consistently similar to North American ducks, and in particular several viruses from sea ducks. This sequence represented the first complete AIV genome from a Thick-billed Murre, the first seabird AIV characterized from eastern North America, and one of few seabird AIV overall with available sequence information (Chapter 2).

In comparison to seabirds, substantially more gull AIV are available, with sequences from a total of 173 viruses in public databases (as of April 2010). In North America, AIV have been predominately isolated from American Herring Gulls (*Larus*

smithsonianus) and Laughing Gulls (*L. atricilla*) (Chapter 4), the latter of which are not found as far north as NL. In this study, viruses were only directly identified in Great Black-backed Gulls (*L. marinus*), regardless of high surveillance effort in American Herring Gulls (Chapter 2). AIV were only found in gulls in NL in the fall, specifically September and October, which is later than viruses have been isolated from gull species in other locations (June – August) (Olsen *et al.* 2006). One virus isolated from a Great Black-backed Gull was characterized, A/Great Black-backed Gull/Newfoundland/296/2008(H13N2), and the genome comprised segments with a mosaic pattern of geographic origins and avian host group relationships (Chapter 3). The segments from this virus were similar to those from Eurasian gulls (PB2, NP, M, and NS), American gulls (PB1 and HA), and American ducks (PA and NA). Based upon BLAST searches and phylogenetic characterization, the PB2, PB1, NP, M and NS segments of this virus were highly similar to a virus isolated from a shorebird in Delaware Bay in 2006, A/shorebird/Delware/168/2006(H16N3). Despite the fact that some of this virus is clearly of Eurasian origin, the high similarity to another virus isolated previously in North America indicate that these Eurasian segments have been circulating in North America for some years. The characterization of this virus also demonstrated that our knowledge of H13 gull viruses is poor. There was a 20 year gap between identification of the HA sequence of A/Great Black-backed Gull/Newfoundland/296/2008(H13N2) and closely related sequences in other AIV. This lineage, predominately including viruses from 1985-1989, is characterized by a 3 base pair insertion, which is absent from all North American H13 viruses sequenced within the last 10 years (Chapter 3).

The available literature suggests that AIV from gulls form different clades than those isolated from other wild bird hosts (Kawaoka *et al*. 1988; Obenauer *et al*. 2006; Dugan *et al*. 2008; Hanson *et al*. 2008), and the hemagglutinin subtypes H13 and H16 have been identified as gull-specific (Hinshaw *et al*. 1982; Yamnikova *et al*. 2003; Foucher *et al*. 2005). Large-scale analyses of intercontinental reassortment have demonstrated a low frequency of reassortment events across all wild bird hosts (Krauss *et al*. 2007; Dugan *et al*. 2008), but analyses have shown that viruses isolated from *Charadriiformes* (i.e. gulls and shorebirds) are the major contributor of such outsider events. Based upon my findings with the NL Great Black-backed Gull AIV, and because there has been no clear delineation of the contribution of gulls, as opposed to shorebirds, to intercontinental AIV exchange, I phylogenetically characterized all available gull virus sequences (Chapter 4). In addition, six viruses isolated in Alaska were sequenced to increase the number of viruses available from the Pacific coast of North America. Due to large spatial, temporal and host species biases in the available data it was not possible to follow lineages through time and space. However, it is evident that there are gull-specific clades, particularly for the NP, M, and NS segments. These clades are less prominent in the polymerase-encoding segments, PB1, PB2, and PA. It is also demonstrated that there are multiple lineages of individual segments circulating within gulls at the locations that contain the largest amount of data, such as Delaware Bay, U.S.A., and the Volga River Delta, Russian Federation. Further investigation of the lineages in Delaware Bay revealed linkage between the PB2, PB1, PA, NP, M, and NS segments and the HA segments over the span of at least 6 years (Chapter 4). Transient linkage has been hypothesized to be

important in AIV dynamics (Chen and Holmes 2010), although some investigations of AIV in waterfowl reveal high levels of reassortment rather than linkage within the same location (Hatchette *et al.* 2004). It has been suggested that all H13 and H16 viruses contain segments with a mosaic pattern of geographic origins (Dugan *et al.* 2008), however there is no intercontinental reassortment in any of the currently known Eurasian H13 and H16 viruses. There was, however, a high frequency of reassortment in the American gull viruses, regardless of hemagglutinin subtype (Chapter 4).

All currently circulating PB1 and PA lineages circulating in North America are of Eurasian descent (Bahl *et al.* 2009). It is hypothesized that these lineages were introduced through geographically reassorted viruses containing at least one segment of Eurasian origin (Bahl *et al.* 2009; Chen and Holmes 2009; Chen and Holmes 2010). This has relevance to the potential introduction of segments from highly pathogenic viruses, such as H5N1, into North America. Alaska has been proposed as an important route for the introduction of these highly pathogenic segments into North America (Peterson *et al.* 2007). The five H13 viruses isolated from Alaska had 7 of 8 segments most similar to viruses isolated in Eurasia, which is the largest number of Eurasian segments found in an American virus to date (Chapter 4). My work suggests that gulls might be important for the introduction of Eurasian genes, both along the Pacific coast (Alaska) and the Atlantic coast (Chapter 3, Chapter 4) and therefore more surveillance effort should be placed upon this family of birds.

Increased surveillance for viruses in underappreciated hosts, such as gulls and seabirds, could dramatically affect our understanding of AIV in wild birds. It has been demonstrated that HPAIV H5N1 has different dynamics than LPAIV (Webster *et al.*

2007), most of which have been isolated from waterfowl. Due to increased fitness of H13 and H16 in gulls, gull-specific clades of other segments, and an increased frequency of geographic reassortment in viruses isolated from gulls, an increase in gull virus sequences could change our understanding of LPAIVs. These observations could indicate that these viruses have different dynamics than AIVs in waterfowl.

As discussed previously, waterfowl are widely believed to be the major hosts of influenza viruses in North America (Webster *et al.* 1992). Newfoundland, particularly eastern Newfoundland where this work was done, is not along the Atlantic Flyway and thus there is not a large seasonal influx of waterfowl. As a result, it might be predicted that NL waterfowl AIV might have different dynamics than those from along large waterfowl migratory corridors. Viruses were only identified in one species of waterfowl in NL, American Black Ducks, in both 2008 and 2009 in one location in St. John's (Chapter 2). Eight viruses isolated in 2008 were characterized, representing the first complete AIV genome sequences from waterfowl in NL. There was variation in these viruses, with single M and NS lineages, two PB2, PB1, and PA lineages, three NP and HA lineages, and four NA subtypes. Overall, the sequences were most similar to viruses isolated from ducks along the eastern coast of North America, following the regional trend of relationships that is often found for AIV (Chen and Holmes 2009). These AIV sequences greatly increase the data available from Atlantic Canada.The eight AIV also greatly increase the number of sequences available from American Black Ducks. Most previous sampling has focused predominately on species known to have high viral prevalence such as Mallards, Northern Pintails, and teals. The genome characteristics of

these viruses were not obviously distinct from what has been found in previous studies of Canadian waterfowl, but do add spatial and host species information.

5.2 Future Work

Continued surveillance and further detection, isolation, and characterization of viruses from murres would contribute greatly to our understanding of AIV in these seabirds. Additionally, a serological study would increase our knowledge about overall AIV infection rates in these species, and if AIV historical infection varies greatly between specific populations or locations. Although the length of time that antibodies against influenza remain in the birds is unknown, a serological study would provide a much broader view of AIV in murres. The surveillance approach employed for my thesis work requires detection of AIV particle release by the birds, and this may only be possible for weeks or days during an infection.

Completion of the 2010 gull dataset, as it has consistent sampling effort throughout the year, would be a great benefit in determining peak viral prevalence in Newfoundland, thus enabling more specific temporal targeting of these birds in the future. Only a single gull virus genome is available from Newfoundland, and therefore further detection, isolation and characterization of viruses would benefit our understanding of virus movement along the east coast of North America. Furthermore, characterization of viruses from other locations in Atlantic Canada, as well as the Arctic, would be beneficial as many of those gull populations spend time in Newfoundland, particularly during the winter.

This study focused on the detection of Eurasian AIV segments in Newfoundland. A study of the AIV in Eurasian species, with known intercontinental and pelagic movements would be beneficial in understanding the emigration of Eurasian AIV segments. Species such as Lesser Black-backed Gulls (*Larus fuscus*), Great Black-backed Gulls (*L. marinus*), Yellow-legged Gull (*L. michahellis)*, Black-headed Gulls (*Chroicocephalus ridibundus*), Black-legged Kittiwakes (*Rissa tridactyla*), from coastal European regions, Glaucous Gulls (*L. hyperboreus*), Glaucous-winged Gulls (*L. glaucescens*), Slatey-backed Gulls (*L. schistisagus*) from coastal Asia, and Ivory Gull (*Pagophila eburnea)*, Iceland Gull (*L. glaucoides)* and Glaucous Gulls (*L. hyperboreus*) from arctic regions would be good candidates. Further, a more detailed study pertaining to specific movements of gulls would allow us to better understand the routes these AIV hosts may travel, and thus identify key locations where the introduction of these Eurasian AIV into North America occurs.

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Appendix 1

Primers used to amplify gull genomes sequences of viruses isolated in Newfoundland and Alaska between Sept 2008-Aug 2010.

Segment Region	Name	Primer	PCR Conditions[a]	Source
All (cDNA)	UNI12M	AGCRAAAGCAGG		[1]
PB2.1	Bm-PB2-1	TATTGGTCTCAGGGAGCGAAAGCAGG TC	2	[2]
	PB2-1250R	TCYTCYTGTGARAAYACCAT		[3]
PB2.2	PB2-1105F	TAYGARGARTTCACAATGGT	1	[3]
	B010 PB2-1962R	ATTCCKGAGCCTCTCACATTCACAG		[4]
PB2.3	B022 PB2.2-1814F	TGAGGACACTGTTCCAGCAGATG	1	[4]
	AVAKPB222R	CAAGGTCGTTTTTAAACAATTCG		[5]
PB1.1	Bm-PB1-1	TATTCGTCTCAGGGAGCGAAAGCAGG CA	2	[2]
	A006R PB1-621R	CATTTTCTTGGTCATGTTG		[4]
PB1.2	A014F PB1-533F	TAGATTTTCTCAAGGATGTGATGGA	2	[4]
	PB1-1262R	TTRAACATGCCCATCATCAT		[3]
PB1.3	PB1-1124F	ARATACCNGCAGARATGCT	2	[3]
	A012 PB1-1964R	GGCATTACCACAGCATTGTT		[4]
PB1.4	A011 PB1-1944F	AACAATGCTGTGGTGATGCC	2	[4]
	PB1-1564	GGAGCTGCCCAGCTTTGGAG	2	[4]
	Bm-PB1-2341R	ATATCGTCTCGTATTAGTAGAAACAA GGCATTT		[2]
PA.1	Bm-PA-1	TATTCGTCTCAGGGAGCGAAAGCAGG TAC	1	[2]
	C006 PA607R	CGGATTGACGAAAGGAGTCCC		[4]
PA2	C005 PA587F	GGGAYTCCTTTCGTCAGTCCG	1	[4]
	PA-1498R	TNGTYCTRCAYTTGCTTATCAT		[3]
PA.3	PA-747F	CATTGAGGGCAAGCTTTC	1	[3]

	C011 PA1919R	GARCCTTCYTCCACYCCYTTRGG		[4]
PA4	C016 PA1897F	CCYAARGGRGTGGARGAAGGYTC	1	[4]
	PA1543	GGARTTYTCYCTYACWGAYCC	1	[4]
	Bm-PA-2233R	ATATCGTCTCGTATTAGTAGAAACAA GGTACTT		[2]
NP.1	SZANPF	CTCGAGAGCAAAAGCAGGGT	1	[6]
	E010 NP-734R	AATTTCCCTTTGAGGATGTTGCACATT C		[4]
NP.2	E003 NP-517F	GGAATGGAYCCCAGGATGTGCTC	1	[4]
	SZANPR	AGTAGAAACAAGGGTATTTTTC		[6]
M	SZAMF	CTCGAGCAAAAGCAGGTAGAT	1	[6]
	SZAMR	ATGAGAAACAAGGTAGTTTTT		[6]
NS	SZANSF	AGCAAAAGCAGGGTGACAAA	1	[6]
	SZANSR	ATGAGAAACAAGGGTGTTTTTT		[6]
HA.1	HAF M	TATTACGCGTCGAGGAGCAAAAGCA GGGG	2	[7]
	HA1134R	CCATACCANCCRTCDATCATTC		[8]
HA.2	HA1134F	CCATACCANCCRTCDATCATTCC	1	[8]
	HA929F	TGGGAGCAATAAATACAAC	1	[4]
	HAR K	ATATGGCGCCGTATTAGTAGAAACAA GGGTGTTTT		[7]
H13.1	HA13intR	TCAATGGCCTTTTGTGTTGA	1	This study[b]
H13.1	HA_av_H13_M13_1F	TGTAAAACGACGGCCAGTAGCAAAA GCAGGGGA	1	[9]
	HA_av_H13_M13_783R	CAGGAAACAGCTATGACCCCARTAAA TYTTCATCCARCTC		[9]
H13.2	HA_av_H13_M13_579F	TGTAAAACGACGGCCAGTGACYTACA ACAAYACHACKGGHAG	1	[9]
	HA_av_H13_M13_1195 R	CAGGAAACAGCTATGACCGTGTYGA YTCTTTRTCTGCAGC		[9]
H13.3	HA_av_H13_M13_621F	TGTAAAACGACGGCCAGTGGGDATA CAYCAYCCWGT	1	[9]

	HA_av_H13_M13_1265R	CAGGAAACAGCTATGACCGARTCATARTTYCCRTTCATTTTDTC		[9]
H13.4	HA_av_H13_M13_1088F	TGTAAAACGACGGCCAGTTTGCAGGVTTCATAGAAGGWGG	1	[9]
	HA_av_H13_M13_1770R	CAGGAAACAGCTATGACCAGTAGAAACAAGGGTRTTT		[9]
H16.1	HA_av_H16_M13_1F	TGTAAAACGACGGCCAGTAGCAAAAGCAGGGGATA	1	[9]
	HA_av_H16_M13_783R	CAGGAAACAGCTATGACCATBARGTGCCAATADAKTTTCATCC		[9]
H16.2	HA_av_H16_M13_591F	TGTAAAACGACGGCCAGTCAGGCAGAGATGTTYTAG	1	[9]
	HA_av_H16_M13_122R	CAGGAAACAGCTATGACCATATTRTTWATYTTTGTTGTTATYTC		[9]
H16.3	HA_av_H16_M13_620F	TGTAAAACGACGGCCAGTATTCACCAYCCDGAYACAG	1	[9]
	HA_av_H16_M13_130R	CAGGAAACAGCTATGACCAKCATGTTGATYCTCTTTTCYA		[9]
H16.4	HA_av_H16_M13_108F	TGTAAAACGACGGCCAGTGGMTTYATAGAAGGAGGRTGG	1	[9]
	HA_av_H16_M13_176R	CAGGAAACAGCTATGACCAGTAGAAACAAGGGTRTTT		[9]
NA.1	NAF M	TATTACGCGTCGAGGGAGCAAAAGCAGGAGT	1	[7]
NA.2	NAR K	ATATGGCGCCGTATTAGTAGAAACAAGGAGTTTTTT	1	[7]
N1	N1.1	GAACAGGCAGTTGTGGTC	3	[10]
	N1.2	TYAGTTCTGGATGCTGGA		[10]
N2	N2.1	TCCGTTTCATTTGGGAAC	3	[10]
	N2.2	CTGACAATGGRCTAATGTG		[10]
N3	N3.1	ATCATGTGAYTCYCCAAG	3	[10]
	N3.2	TCCCGATCCAGGTTCAT		[10]
N4	N4.1	ATGTGCATGCAACAGGGTTC	3	[10]
	N4.2	CTGTTGTCTCYCCTCTAATGC		[10]

N5	N5.1	AYCCTGCAACACCACTGAG	3	[10]
	N5.2	TCTCTTTCATTTGTCACCAT		[10]
N6	N6.1	AACCGGAGGGAGCCCAGATC	3	[10]
	N6.2	TCCCAATCGCTCYTTGGATC		[10]
N7	N7.1	ATGYTGAARATACCYAATGC	3	[10]
	N7.2	ARGAACCRGAACCAACTG		[10]
N8	N8.1	ACAGTCRTTAGGGAATAC	3	[10]
	N8.2	TACACATTGGGTGATG		[10]
N9	N9.1	TGTAATGACCCTTATCCAGG	3	[10]
	N9.2	GTTCCATTGTCCAAGGAATTC		[10]

[a] Reactions contained 0.5 µl cDNA, 0.3 mM $MgCl_2$, 0.2 mM each dNTP (New England Biolabs), 0.2 mM of each primer, and 0.5 U Platinum Taq DNA Polymerase (Invitrogen) in a final volume of 25 µl 1x PCR Buffer (Invitrogen).

1. Thermocycler conditions: 2 min at 94°C, followed by 40 cycles of 94°C for 30s, 55°C for 30s, 72°C for 2min, followed by 72°C for 10min.

2. Thermocycler conditions: 2 min at 94°C, followed by 40 cycles of 94°C for 30s, 52°C for 30s, 72°C for 2min, followed by 72°C for 10min.

3. Thermocycler conditions: 2 min at 94°C, followed by 40 cycles of 94°C for 40s, 60°C for 40s, 72°C for 40s, followed by 72°C for 10min.

[b] Primer used in sequencing of A/Great Black-backed Gull/Newfoundland/296/2008(H13N2) only

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Appendix 2

Reference sequences included in construction of phylogenetic trees

Virus Name	Segment GenBank Accession Number					
	Clade[a]					
	PB2	PB1	PA	NP	M	NS
A/blue winged teal/TX/75/2002(H1N3)	FJ357052	FJ357051	FJ357050	FJ357048	FJ357046	FJ375049
	AmAv	AmAv	AmAv	AmAv	AmAv	AmAv
A/blue-winged teal/ALB/293/1994 (H4N6)	CY004910	CY004909	CY004908	CY004906	CY004904	CY004907
	AmAv	AmAv	AmAv	AmAv	AmAv	AmAv
A/mallard/Alberta/209/2003(H10N7)	CY004359	CY004358	CY004357	CY004355	CY004353	CY004357
	AmAv	AmAv	AmAv	AmAv	AmAv	AmAv
A/mallard/California/7766/2008(H4N6)	CY039736	CY039737	CY039738	CY039740	CY039742	CY039743
	AmAv	AmAv	AmAv	AmAv	AmAv	AmAv
A/mallard/Ohio/56/1999(H1N1)	CY012831	CY012830	CY012829	CY012827	CY012825	CY012828
	AmAv	AmAv	AmAv	AmAv	AmAv	AmAv
A/Northern shoveler/NC/6412-	GU186481	GU186480	GU186479	GU186477	GU186475	GU186478
052/2005(H7N6)	AmAv	AmAv	AmAv	AmAv	AmAv	AmAv
A/pintail/Alaska/310/2005(H4N6)	CY017748	CY017747	CY017746	CY017744	CY017742	CY017745
	AmAv	AmAv	AmAv	AmAv	AmAv	AmAv
A/pintail/Ohio/454/1987(H3N8)	CY016147	CY016146	CY016145	CY016143	CY016141	CY016144
	AmAv	AmAv	AmAv	AmAv	AmAv	AmAv
A/shoveler/ALB/114/1985(H6N2)	CY004225	CY004224	CY004223	CY004221	CY004219	CY004222
	AmAv	AmAv	AmAv	AmAv	AmAv	AmAv

A/ruddy turnstone/DE/773/1988 (H9N6)	CY004574	CY004573	CY004572	CY004570	CY004568	CY004571
	AmAv	AmAv	AmAv	AmAv	AmAv	AmAv
A/ruddy	GU050954	GU050953	GU050952	GU050950	GU050948	GU050951
turnstone/Delaware/1057/2001(H10N7)	AmAv	AmAv	AmAv	AmAv	AmAv	AmAv
A/Duck/Hokkaido/8/80 (H3N8)	AB274963	AB274964	AB274965	AB274984	AB274986	AB274987
	EurAv	EurAv	EurAv	EurAv	EurAv	EurAv
A/duck/Hubei/2911/2007(H5N1)	FJ784836	FJ784820	FJ784804	FJ784788	FJ784884	FJ784868
	EurAv	EurAv	EurAv	EurAv	EurAv	EurAv
A/duck/Nanchang/1681/1992(H3N8)	CY005475	CY005474	CY005473	CY005471	CY005469	CY005472
	EurAv	EurAv	EurAv	EurAv	EurAv	EurAv
A/mallard/ZhaLong/88/2004(H4N6)	FJ349254	FJ349253	FJ349252	FJ349249	FJ349251	FJ349250
	EurAv	EurAv	EurAv	EurAv	EurAv	EurAv
A/ruddy	GQ907341	GQ907340	GQ907339	GQ907337	GQ907335	GQ907338
shelduck/Mongolia/37/2005(H12N3)	EurAv	EurAv	EurAv	EurAv	EurAv	EurAv
A/duck/Novosibirsk/02/05(H5N1)[b]	DQ864713	DQ864714	DQ864715	DQ864709	DQ864708	DQ864707
	EurAv	EurAv	EurAv	EurAv	EurAv	EurAv
A/Eurasian	CY043863	CY043862	CY043861	CY043859	CY043857	CY043860
wigeon/Netherlands/3/2005(H9N2)	EurAv	EurAv	EurAv	EurAv	EurAv	EurAv
A/greylag goose/Netherlands/4/1999(H6N1)	CY060195	CY060196	CY060197	CY060199	CY060201	CY060202
	EurAv	EurAv	EurAv	EurAv	EurAv	EurAv
A/mallard/Marquenterre/Z237/1983(H1N1)	DQ864507	DQ864502	DQ864508	DQ864509	GU066781	GU066782
	EurAv	EurAv	EurAv	EurAv	EurAv	EurAv
A/mallard/Sweden/6/2002(H2N3)	CY060363	CY060364	CY060365	CY060367	CY060369	CY060370
	EurAv	EurAv	EurAv	EurAv	EurAv	EurAv

A/shorebird/Delaware/68/2004(H13N9)[c]	CY004450 EurGull	CY004449 AmGull				CY004447 AmGull
A/shorebird/Delaware/221/2006(H13N9)[c]	CY043895 AmGull	CY043894 AmGull	CY043893 EurGull			
A/shorebird/Delaware/168/06(H16N3)[c]	EU030974 EurGull	EU030973 AmGull	EU030975 EurGull	EU030977 EurGull	EU030979 EurGull	EU030980 EurGull
A/shorebird/Delaware/224/2006(H13N9)[c]					CY043897 EurGull	
A/shorebird/Delaware/224/1997(H16N6)[c]		CY015151 AmGull	CY015150 AmGull		CY015147 AmGull	CY015149 AmGull
A/shorebird/NJ/840/1986(H13N3)[c]				CY005372 AmGull		
A/laughing gull/New Jersey/Sg-00485/2008(H13N9)[c]	CY042427 EurGull					
A/laughing gull/New Jersey/Sg-00559/2008(H13N9)[c]		CY042591 AmGull	CY042592 EurGull			
A/gull/Maryland/704/1977(H13N6)[c]	CY014701 EurGull	CY014700 AmGull	CY014699 AmGull	CY01469 AmGull	CY014695 EurGull	CY01498 AmGull
A/gull/Minnesota/945/1980(H13N9)[c]	CY005865 AmGull	CY005864 AmGull	CY005863 AmGull	CY005861 AmGull	CY005859 AmGull	CY005862 AmGull
A/gull/Massachusetts/26/1980(H13N6)[c]		GU051932 AmGull	GU05193 AmGull 1	M30752 AmGull		U96744 AmGull
A/laughing gull/DE/2838/1987 (H13N2)[c]	CY005071 AmGull	CY005070 AmGull	CY005069 AmGull	CY005067 AmGull	CY005065/ AmGull	CY005068 AmGull

A/herring gull/DE/712/1988 (H16N3)[c]	CY004567 AmGull	CY004566 AmGull	CY004565 AmGull	CY004563 AmGull	CY004562 AmGull	CY004564 AmGull
A/herring gull/NJ/782/1986 (H13N2)[c]	CY004457 AmGull	CY004456 AmGull	CY004455 AmGull	CY004453 AmGull	CY004451 AmGull	CY004454 AmGull
A/laughing gull/DE/554/1988(H13N3)[c]		CY005386 AmGull	CY005385 AmGull	CY005383 AmGull	CY005382 AmGull	CY005384 AmGull
A/herring gull/DE/660/1988(H13N6)[c]		CY005391 AmGull	CY005390 AmGull	CY005388 AmGull	CY005388 AmGull	CY005389 AmGull
A/herring gull/DE/475/1986(H13N2)[c]	CY003901 AmGull	CY003900 AmGull	CY003899 AmGull	CY003897 AmGull	CY003895 AmGull	CY003989 AmGull
A/gull/Maryland/1815/1979(H13N9)[c]				M30756 AmGull	M30756 AmGull	
A/duck/Siberia/272/1998(H13N6)[c]	AB284985 EurGull	Ab284986 EurGull	AB284987 EurGull	AB284989 EurGull	AB284991 EurGull	AB284090 EurGull
A/black-headed gull/Sweden/1/99 (H16N3)[c]	AY684875 EurGull	AY684879 EurGull	AY684883 EurGull	AY684897 EurGull	AY684907 EurGull	
A/black-headed gull/Sweden/2/99 (H16N3)[c]	AY684876 EurGull		AY684884 EurGull	AY684898 EurGull	AY684908 EurGull	AY684912 EurGull
A/black-headed gull/Sweden/3/99 (H16N3)[c]						AY684913 EurGull
A/black-headed gull/Sweden/5/99 (H16N3)[c]		AY684881 EurGull	AY684885 EurGull	AY684899 EurGull	AY684909 EurGull	
A/black-headed gull/Netherlands/1/00(H13N8)[c]	AY684874 EurGull	AY684878 EurGull	AY684882 EurGull	AY684896 EurGull	AY684906 EurGull	AY684910 EurGull

A/gull/Astrakhan/227/1984(H13N6)[c]	M73516[c]	M30753 EurGull		
A/gull/Astrakhan/1846/1998(H13N6)[c]			EU580591 EurGull	
A/black-headed gull/Astrakhan/65/1983 (H13N6)[c]			EU580589 EurGull	
A/gull/Astrakhan/44/1988(H13N6)[c]				EU580578 EurGull

[a] sequenes are given a clade based upon position in trees presented in Figure 1 of the main text where AmAv is the American Avian clade, EurAv is the Eurasian Avian clade, AmGull is the American Gull clade and EurGull is the Eurasian Gull clade.

[b]Sequences used as the outgroup for Bayesian trees in Chapter 3

[c]These sequences were used as the construction of phylogenetic trees in Chapter 3 only.

Appendix 5

Locations and coordinates of all locations in Newfound and Labrador from which birds were captured and fecal deposits collected for AIV sampling.

Location Name	Coordiantes
Gull Island, Witless Bay Ecological Reserve	47°15'34"N, 52°46'26"W
Great Island, Witless Bay Ecological Reserve	47°10'58.90"N, 52°48'28.55"W
Little Bell Island	47°33'56.92"N, 52°58'28.49"W
Kelly's Island	47°32'29.48"N, 53°0'21.98"W
Cape St. Mary's	46°49'14.37"N, 54°11'39.53"W
Baccalieu Island	48°7'0.37"N, 52°47'38.66"W
Middle Lawn Island	46°52'8.78"N, 55°37'2.88"W
Funk Island	49°45'7.42"N, 53°11'31.13"W
Gannet Islands	53°56'21.22"N, 56°32'45.43"W
Robin Hood Bay Landfill, St. John's	47°36'11.90"N, 52°40'18.34"W
Logy Bay, St. John's	47°37'31.45"N, 52°39'38.95"W
Quidi Vidi Lake, St. John's	47°34'42.18"N, 52°41'59.18"W
Mundy Pond, St. John's	47°33'8.09"N, 52°44'23.12"W
Commonwealth Pond, St. John's	47°30'2.54"N, 52°47'22.23"W
Burton's Pond, St. John's	47°34'25.20"N, 52°43'42.35"W
"Bubble"/US Navy Dock, St. John's	47°34'12.37"N, 52°41'50.46"W
Signal Hill Trail, St. John's	47°34'5.59"N, 52°40'55.21"W
Fort Amherst Small Boat Harbour, St. John's	47°33'56.18"N, 52°41'21.19"W

Appendix 6

Viruses detected from wild birds in Newfoundland and Labrador from birds 2007-June 2010

Virus Name	Host Species	Age[1]	Sex[2]	Date[3]	Notes[4]
A/Thick-billed Murre/ Newfoundland/031/2007 (H11N2)	Thick-billed Murre	SY	U	6 April 2007	Mortality event[5]
A/Thick-billed Murre/ Newfoundland/040/2007	Thick-billed Murre	ASY	U	6 April 2007	Mortality event[5]
A/Thick-billed Murre/ Newfoundland/108/2010	Thick-billed Murre	SY	U	14 Feb 2010	Hunt
A/Common Murre/ Newfoundland/363/2010	Common Murre	U	U	3 June 2010	Fecal sample
A/Great Black-backed Gull/Newfoundland/0296/2008 (H13N2)	Great Black-backed Gull	HY	U	19 Oct 2008	Dead bird
A/Great Black-backed Gull/Newfoundland/0355/2008	Great Black-backed Gull	ATY	U	20 Oct 2008	Dead bird
A/gull/Newfoundland/1413/2009	Ring-billed Gull or Herring Gull	U	U	3 Sept 2009	Mixed flock fecal sample
A/Great Black-backed Gull/Newfoundland/1426/2009	Great Black-backed Gull	U	U	19 Sept 2009	GBBG fecal sample[6]

A/Great Black-backed Gull/Newfoundland/1432/2009	Great Black-backed Gull	U	U	9 Sept 2009	GBBG fecal sample[6]
A/Great Black-backed Gull/Newfoundland/1435/2009	Great Black-backed Gull	U	U	9 Sept 2009	GBBG fecal sample[6]
A/gull/Newfoundland/1444/2009	Great Black-backed Gull or Herring Gull	U	U	9 Sept 2009	Mixed flock fecal sample
A/Great Black-backed Gull/Newfoundland/MW172/2010	Great Black-backed Gull	U	U	22 Feb 2010	GBBG fecal sample[6]
A/Great Black-backed Gull/Newfoundland/MW174/2010	Great Black-backed Gull	U	U	22 Feb 2010	GBBG fecal sample[6]
A/American Black Duck/Newfoundland/732/2008(H3)	American Black Duck	HY	M	15 Sept 2008	H3
A/American Black Duck/Newfoundland/734/2008(H3 N8)	American Black Duck	HY	M	15 Sept 2008	H3
A/American Black Duck/Newfoundland/797/2008	American Black Duck	HY	M	19 Sept 2008	n/a[7]
A/American Black Duck/Newfoundland/812/2008(H2)	American Black Duck	HY	M	26 Sept 2008	H2

A/American Black Duck/Newfoundland/819/2008(H2N6)	American Black Duck	HY	M	26 Sept 2008	H2
A/American Black Duck/Newfoundland/807/2008(H4N4)	American Black Duck	HY	M	26 Sept 2008	H4
A/American Black Duck/Newfoundland/826/2008(H4)	American Black Duck	HY	M	1 Oct 2008	H4
A/American Black Duck/Newfoundland/836/2008(H2N4)	American Black Duck	HY	M	8 Oct 2008	H2
A/American Black Duck/Newfoundland/840/2008(H2N4)	American Black Duck	HY	M	8 Oct 2008	H2
A/American Black Duck/Newfoundland/865/2008(H4)	American Black Duck	HY	M	15 Oct 2008	H4
A/American Black Duck/Newfoundland/1146/2009	American Black Duck	HY	M	25 Sept 2009	n/a[7]
A/American Black Duck/Newfoundland/1148/2009	American Black Duck	HY	F	25 Sept 2009	n/a[7]

A/American Black Duck/Newfoundland/1150/2009	American Black Duck	HY	M	25 Sept 2009	n/a[7]
A/American Black Duck/Newfoundland/1153/2009	American Black Duck	AHY	U	25 Sept 2009	n/a[7]
A/American Black Duck/Newfoundland/1169/2009(H1)	American Black Duck	HY	M	30 Sept 2009	H1
A/American Black Duck/Newfoundland/1181/2009		HY	M	13 Oct 2009	n/a[7]

[1] The age group of the bird as defined by bird banding protocols: HY is the period from hatch to the end of calendar year, SY is the second calendar year of life, AHY, ASY and ATY is for adult birds, and U indicates age is unknown.

[2] The sex categories of birds: M is for Male, F is for female, U indicates sex is unknown

[3.] Date birds were recovered.

[4] Notes include information about the circumstances under which seabird and gull samples were collected or subtypes of viruses (ducks)

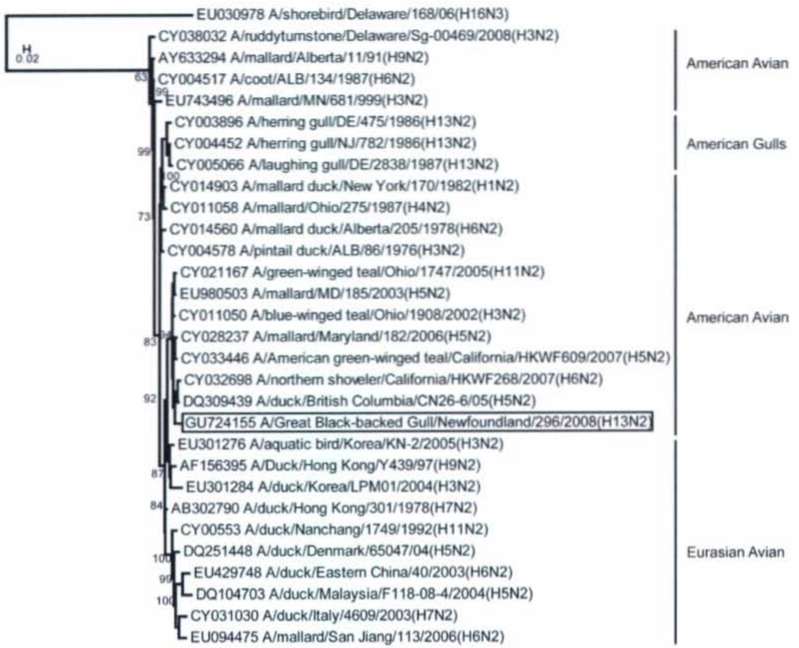
[5] Birds collected during a mass mortality event attributed to starvation (McFarlane Tranquilla *et al.* 2010).

[6] Sample from roosting site used exclusively by Great Black-backed Gulls

[7]No subtype information could be obtained by RT-PCR

Appendix 6

Phylogenetic analysis of the NA gene of A/Great Black-backed Gull/Newfoundland/296/2008(H13N2) from Chapter 3



The Bayesian inference tree is rooted with A/influenza/shorebird/Delaware/168/06(H16N3). A/Great Black-backed Gull/Newfoundland/296/2008(H13N2) is indicated by a box. Bayesian posterior probabilities are indicated as percentages. The scale bar indicates the expected number of nucleotide substitutions per site. Sequences from the American avian, American gull and Eurasian avian clades are indicated

Appendix 6

Accession numbers of gull viruses available in GenBank as of April 2010

Virus Name	Segment							
	PB2	PB1	PA	HA	NP	NA	M	NS
A/gull/01.19/1978(H10N4)							GQ404606	
A/gull/Delaware/2952/1988(H11N4)						AY207545		M80978
A/ring-billed gull/Georgia/2658/2000(H11N6)							GU051386	GU051085
A/ring-billed gull/Georgia/2667/2000(H11N6)		GU051089	GU050869		GU050868		GU050867	GU051088
A/gull/Maryland/5/1977(H11N9)					M63777			
A/laughing gull/DE/94/2000(H12N4)	CY005364	CY005363	CY005362	CY014598	CY005360	CY005359.1	CY005358	CY005361
A/herring gull/Germany/wv1136KRK/03(H13)				AM087221				
A/Larus ridibundus/Germany/R2064/2006(H13)				AM922164				
A/yellow-legged gull/Ukraine/912306/2005(H13)				EU599306				EU599307
A/Great Black-backed Gull/Newfoundland/296/2008(H13N2)	GU724150 This study	GU724151 This study	GU724152 This study	GU724153 This study	GU724154 This study	GU724155 This study	GU724156 This study	GU724157 This study
A/great black-headed gull/Astrakn/1420/79(H13N2)				EU293858				

A/great black-headed gull/Astrakn/591/82(H13N2)				EU293860				
A/great black-headed gull/Gurjev/76/83(H13N2)				EU293861				
A/gull/Astrakn/176/1986(H13N2)				EU835899				
A/herring gull/DE/475/1986(H13N2)	CY003901	CY003900	CY003899	CY005914	CY003897	CY003896	CY003895	CY003989/M80956
A/herring gull/NJ/782/1986(H13N2)	CY004457	CY004456	CY004455	CY005932	CY004453	CY004452	AY664475/CY004451	CY004454
A/Larus ichthyaetus/Astrakn/75/1983(H13N2)				EU564107				
A/laughing gull/DE/2838/1987(H13N2)	CY005071	CY005070	CY005069	CY005979	CY005067	CY005066	CY005065/AY664427	CY005068
A/laughing gull/DE/554/1988(H13N3)		CY005386	CY005385		CY005383		CY005382	CY005384
A/laughing gull/New Jersey/171/92(H13N5)				AF310992				
A/black-headed gull/Mongolia/1766/2006(H13N6)	GQ907309	GQ907308	GQ907307	GQ907302	GQ907305	GQ907304	GQ907303	GQ907306
A/black-headed gull/Sweden/1/99(H13N6)	AY684875	AY684879	AY684883	AY684887	AY684897	AY684901	AY684907	AY684911

A/blackhead gull/Astrakan/1421/1979(H13N6) or A/great black-headed gull/Astrakan/1421/1979(H13N6)	GU052239	GU052238	Gu052237	EU293859	GU052235		GU052234	GU052236
A/Larus ichthyaetus/Astrakn/44/1988(H13N6)	GU052252	GU052251	GU052250	EU564115	GU052248	EU580569/GU052247	GU052246	EU580578/GU052249
A/blackhead gull/Astrakhan/458/1985(H13N6)	GU052245	GU052244	GU052237		GU052241		GU052240	EU580579/GU052242
A/blackhead gull/Astrakhan/65/1983(H13N6)	GU052228	GU052227	GU052237	EU580577	GU052224		EU580589/GU052223	GU052225/EU580580
A/gul/Astrakn/1314/1979(H13N2)				EU835898				
A/gull/Astrakhan/1818/1998(H13N6)				EU835900				
A/gull/Astrakhan/1846/1998(H13N6)	GU052233	GU052234		EU580576	GU052230		GU052229/EU58059*	GU052231
A/gull/Astrakhan/226/1984(H13N6)				EU835895				
A/gull/Astrakhan/227/1984(H13N6) or A/blackhead gull/Astrakhan/227/1984(H13N6)	M73516			M26089	M30753	AY207552		
A/gull/Astrakhan/3483/2002(H13N6)				EU835897				
A/gull/Astrakhan/998/1990(H13N6)				EU835896				
A/glaucous-winged gull/Southcentral_Alaska/ 9JR0691R1/2009(H13N6)	CY070847 This study	CY070848 This study	CY070849 This study	CY070850 This study	CY070851 This study	CY070852 This study	CY070853 This study	CY070854 This study

A/glaucous-winged gull/Southcentral_Alaska/ 9JR0738R1/2009(H13N6)	CY070855 This study	CY070856 This study	CY070857 This study	CY070858 This study	CY070859 This study	CY070860 This study	CY070861 This study	CY070862 This study
A/glaucous-winged gull/Southcentral_Alaska/ 9JR0747R1/2009(H13N6)	CY070863 This study	CY070864 This study	CY070865 This study	CY070866 This study	CY070867 This study	CY070868 This study	CY070869 This study	CY070870 This study
A/gull/Stralsund/Wv1136-40/03(H13N6)				AM922163				
A/herring gull/Astrakn/458/85(H13N6)				EU293863		EU580570		
A/herring gull/Astrakn/479/85(H13N6)				EU293862		EU580570.1		
A/glaucous-winged gull/Southcentral_Alaska/ 9JR0769R1/2009(H13N6)	CY070871 This study	CY070872 This study	CY070873 This study	CY070874 This study	CY070875 This study	CY070876 This study	CY070877 This study	CY070878 This study
A/herring gull/Norway/10_2336/2006(H13N6)				FM179758	EU564106			
A/Larus ichthyaetus/Astrakn/10/1988(H13N6)								
A/Larus minutus/Astrakn/3357/2002(H13N6)				EU564108				
A/Mongolian gull/Mongolia/401/2007(H13N6)	GQ907317	GQ907316	GQ907315	GQ907310	GQ907313	GQ907312	GQ907311	GQ907314
A/Mongolian gull/Mongolia/405/2007(H13N6)	GQ907325	GQ907324	GQ907323	GQ907318	GQ907321	GQ907320	GQ907319	GQ907322

A/glaucous-winged gull/SouthcentralAlaska/9JR0781R1/2009(H13N6)	CY070879 This study	CY070880 This study	CY070881 This study	CY070882 This study	CY070883 This study	CY070884 This study	CY070885 This study	CY070886 This study
A/gull/Maryland/704/1977H13N6	CY014701/M73535	CY014700/GU052017/M25933	CY014699/M26088/CY014699	AB292664/CY014694/D90308/GQ247866/M26090	CY014697/GU052016/M27521	AB292665/CY014696/GQ247867/K01029/AY207553	CY014695/GU052015	CY01498/m80959/U96737
A/gull/Massachusetts/26/1980(H13N6)		GU051932	GU051931	K00383	M30752	GU051930/K01027	M63538	U96744
A/gull/Minnesota/1352/1981(H13N6)								M80958
A/herring gull/DE/660/1988(H13N6)		CY005391	CY005390	CY014603	CY005388	CY005387		CY005389
A/great black headed gull/Atyrau/2966/2008/H13N6				GU953282	GU953280	GU953287	GU953279	GU953281
A/herringgull/Atyrau/280/2002/H13N6				GU953278	GU953276		GU953275	GU953277
A/greatblackheadedgull/Atyrau/742/2004/H13N6						GU953289		
A/greatblack-headedgull/Atyrau/773/2004/H13N6				GU982284	GU982292	GU982288		
A/greatblack-headedgull/Atyrau/767/2004/H13N6				GU982283	GU982291	GU982287		
A/greatblack-headedgull/Atyrau/744/2004/H13N6				GU982282	GU982290	GU982286		

A/greatblack-headedgull/Atyrau/743/2004/H13N6				GU982281	GU982289	GU982285		
A/black-headed gull/Netherlands/1/00(H13N8)	AY684874	AY684978	AY684882	AY684886	AY684896	AY684900	AY684906	AY684910
A/black-headedgull/Norway/10_1459/2006H13N8						FM179764		
A/gull/Maryland/1815/1979(H13N9)					M30756		M63530	
A/gull/Maryland/1824/1978(H13N9)			GU052018		M30755		M63539	U96743
A/gull/Minnesota/945/1980(H13N9)	CY005865	CY005864	CY005863	CY014720	CY005861M30757	CY005860	CY005859	CY005862/U96738
A/kelp gull/Argentina/LDC4/2006(H13N9)	EU523143	EU523142	EU523141	EU523136	EU523140	EU523137	EU523138	EU523139
A/laughing gull/New Jersey/Sg-00485/2008(H13N9)	CY042427	CY042428		CY042429		CY042430		
A/laughing gull/New Jersey/Sg-00559/2008(H13N9)	CY042590	CY042591	CY042592	CY042593		CY042594		
A/laughing gull/New Jersey/Sg-00568/2008(H13N9)	CY042604	CY042605	CY042606	CY042607		CY042608		
A/herring gull/Astrakn/267/1982(H14N5)				FJ975075				
A/glaucous-winged gull/Southcentral Alaska/9JR0783R1/2009(H16N3)	CY070887 This study	CY070888 This study	CY070889 This study	CY070890 This study	CY070891 This study	CY070892 This study	CY070893 This study	CY070894 This study

A/black-headed gull/Mongolia/1756/2006(H16N3)	GQ907301	GQ907300	GQ907299	GQ907294	GQ907297	GQ907296	GQ907295	GQ907298
A/black-headed gull/Sweden/2/99(H16N3)	AY684876	AY684880	AY684884	AY684888	AY684898	AY684902	AY684908	AY684912
A/black-headed gull/Turkmanistan/13/76(H16N3)				EU293864				
A/black-heaed gull/Sweden/5/99(H16N3)	Ay684877	AY684881	AY684885	AY684891	AY684899	AY684905	AY684909	AY684913
A/common gull/Norway/10_1617/2006(H16N3)				FM179755		FM179759		
A/gull/Denmark/68110/2002(H16N3)				GQ247872		GQ247873		
A/herring gull/Norway/10_1623/2006(H16N3)				FM179756		FM179761		
A/slender-billed gull/Astrakn/28/76(H16N3)				EU293865				
A/black-legged kittiwake/Alaska/295/1975(H16N3)			CY015162	CY015160			CY015161	
A/herring gull/DE/712/1988(H16N3)	CY004567	CY004566	CY004565	CY005933	CY004563	CY014569	CY004562	CY004564
A/herring gull/Atyrau/2216/2007/H16N3				GU953286	GU953284		GU953283	GU953285
A/black-headed gull/Sweden/3/99(H16N3)				AY684889		AY684903		

A/black-headed gull/Sweden/4/99(H16N3)				AY684890		AY684904		
A/herring gull/NewJersey/780/86(H1N3)	CY004389				CY004388		AY664422/CY004387	
A/laughinggull/Delaware/34/94H2N3						AY207516		
A/herring gull/DE/698/1988(H2N1)	CY003913	CY004033	CY003912	CY003907	CY003910	CY003909.1	CY003908	CY003911
A/herring gull/Delaware/471/86(H2N7)	CY014602	CY005381	CY005380	CY014601	CY005378	CY005377.1	CY005376	M80957/CY005379
A/laughing gull/NJ/798/1986(H2N7)	CY003929	CY003928	CY003927	CY003922	CY003925	CY003924.1	cy003923/AY664471	CY003926
A/herring gull/DE/677/1988(H2N8)	EU743194	EU743193	EU743192	GU186622/L11132	EU743191	GU186623.1/L06585.1	EU743190	U96745
A/herring gull/DE/692/1988(H2N8)	CY004561	CY004560	CY004559	CY004554	CY004457		CY004555	CY004558
A/herring gull/DE/703/1988(H2N8)	CY003921	CY003920	CY003919	CY003914	CY003917	CY003916.1	CY00915	CY003918
A/gull/MD/19/1977(H2N9)	EU742651/CY005813	EU742650/CY005812	EU742649/CY005811	L11130/CY005808/EU742644	EU742647/CY005809	CY014647.1/EU742646.1	EU742645/CY014646	EU74222222 2648/U96742 /CY005810
A/herring gull/DE/670/1988(H2N9)	CY003906	CY003905	CY003904	CY014556	CY014557		CY003902	CY003903
A/laughing gull/NJ/75/1985(H2N9)	CY003870	CY003869	CY003868	CY003863/AF116201	CY003866	CY004826	CY003864/AY664437	CY003867
A/gull/36/1977(H3N6)							GQ404605	
A/laughing gull/NJ/768/2005(H3N8)	GU186473	GU186472	GU186471	GU186466	GU186469	GU186468.1	GU186467	GU186470
A/herring gull/DE/665/1998(H4N6)	CY004880	CY004879			CY004877	CY004876.1	CY004875	CY004878

A/laughing gull/NJ/72/1985(H4N9)	CY004830	CY004829	CY004828			CY004826.1	CY004825	CY004827
A/black-headed gull/HK/12.1/2003/H5N1	AY651743	AY651689	AY651635*	AY651373	AY651523	AY651470.1	AY651413	AY651577
A/black-headed gull/Qingan/1/2005(H5N1)	DQ100544	DQ100548	DQ100552	DQ100556	DQ100560	DQ100564.1	DQ100568	DQ100572
A/black-headed gull/Qinghan/3/2006(H5N1)	DQ822558	DQ822555	DQ822552	DQ822564	DQ822549	DQ822561	DQ822546	DQ822543
A/black-heaed gull/Tyva/115/2009(H5N1)				GQ338084		GQ338085		
A/brown-headed gull/Qinghai/03/05(H5N1)	DQ095756	DQ095736	DQ095716	DQ095616	DQ095676	DQ095656	DQ95636	DQ095696
A/brown-headed gull/Qinghai/03/07(H5N1)				FJ602806		FJ602842		
A/brown-headed gull/Qinghai/04/07(H5N1)				FJ602807		FJ602843		
A/brown-headed gull/Qinghai/06/07(H5N1)				FJ602867		FJ602845		
A/brown-headed gull/Thailand/VSMU-28-SPK/2005(H5N1)	EU716168	EU716169		EF178528	EU716170	EF178529	EU716171	
A/brown-headed gull/Thailand/VSMU-4/2008(H5N1)	EU676322	EU676323	EU676324	EU676325		EU676327	EU676328	EU676329
A/common gull/Chany/P/2008 (H5N1)	EU871943	EU871939	EU871942	EU871935	EU871941	EU871937	EU871940	EU871938

A/Great Black-headed gull/Qinghai/12/2007(H5N1)				FJ602814		FJ602851		
A/Great Black-headed gull/Qinghai/2/2005(H5N1)	DQ09754	DQ095734	DQ095714	DQ095614	DQ095674	DQ095654	DQ095634	DQ095694
A/Great Black-headed gull/Qinghai/2/2007(H5N1)				FJ461726				
A/Great Black-headed gull/Qinghai/8/2007(H5N1)				FJ602810		FJ602847		
A/great black-headed gull/Qinghan/1/2005(H5N1)	DQ100545	DQ100549	DQ100553	DQ100557	DQ100561	DQ100565	DQ100569	DQ100573
A/gull/Germany/R882/06(H5N1)				AM408215		AM403148		
A/herring gull/Sweden/V1116/06(H5N1)	EU889046	EU889056	EU889070	EU889078	EU889089	EU889100	EU889109	EU122019
A/slatey-backed gull/Shandong/38/04(H5N1)	DQ835807	DQ835798	DQ835791	DQ188908	DQ835783	DQ188910	DQ835777	DQ835801
A/gull/Pennsylvania/4175/83(H5N1)				AF082043			DQ107466	
A/herring gull/NJ/402/1989(H5N3)	CY004398	CY004397	CY004396	CY005926	CY004394	CY004393	CY004392/AY664440	CY004395
A/herring gull/NJ/406/1989(H5N3)	CY004976	CY004975	CY004974		CY004973	CY004972		
A/Sabines gull/Alaska/296/1975(H5N3)			CY015159			CY015157	CY015156	CY015158
A/gull/Delaware/4/2000(H5N4)							DQ107458	
A/gull/Delaware/5/2000(H5N4)							DQ107459	
A/herring gull/Delaware/281/98(H5N8)							AY664452	

A/glaucous gull/Wisconsin/486108-4/2007(H5N9)	GU051993	GU051992	GU051991		GU051989		GU051988	GU051990
A/ring-billed gull/DE/421733/01(H6)				DQ021670	DQ021819		DQ021750	DQ021582
A/gull/Maryland/4/77H6N1						AY207544		
A/gull/Moscow/3100/2006(H6N2)	EU152234	EU152234	EU152236	EU152237	EU152238	EU152239	EU152240	EU152241
A/gull/Delaware/18/2000(H6N4)							DQ107415	
A/laughing gull/New York/470/2000(H6N4)		GU051382	GU051381	DQ021649	DQ021784		DQ021690	DQ021590
A/ring-billed gull/GA/421733/2001(H6N4)				GQ117282				
A/ring-billed gull/Georgia/124/2001(H6N4)	GU051389	GU051388	GU051387					
A/black-headed gull/Netherlands/1/2005(H6N8)	CY041385	CY041384	CY041383	CY041378	CY041381	CY041380	CY041379	CY041382
A/laughing gull/NJ/276/1989(H6N8)	CY004101	CY004100	CY004099	CY004094	CY004097	CY004096	CY004095	CY004098
A/gull/Italy/692-2/93(H7N2)				AF202248				
A/gull/Heuwiese/899-6/80H7N3						AY207515		
A/laughing gull/Delaware Bay/46/2006(H7N3)	CY037094	CY037093	CY037092	CY037087	CY037090	CY037089	CY037088	CY037091
A/laughing gull/Delaware Bay/6/2006(H7N3)	CY037086	CY037085	CY037084	CY037079	CY037082	CY037081	CY037080	CY037083
A/laughing gull/Delaware/2455/2000(H7N3)	GU051494	GU051493	GU051492		GU051491	GU051490		

A/laughing gull/Delaware/42/06(H7N3)	EU030982	EU030981	EU030983	EU030984	EU030985	EU030986	EU030987	EU030988
A/laughing gull/NY/2455/00(H7N3)							DQ021689	DQ021641
A/gull/Shimane/91/88(H7N8)				AB270593				
A/laughing gull/DE/5/2003(H9N1)	CY004427	CY004426	CY004425	CY004420	CY004423	CY004422	CY004421	CY004424
A/Mediterranean gull/Camargue/063353/2006(H9N2)				EU333949		EU333950		
A/laughing gull/Delaware/12/2006(H9N2)	CY041433	CY041432	CY041431	CY041426	CY041429	CY041428.1	CY041427	CY041430
A/laughing gull/DE/2718/1987(H9N5)	CY005126	CY005125	CY005124	CY005986	CY005122	CY014590.1	CY005121	CY005123

Appendix 7

Virus sequences included in the H13/H16 phylogenetic tree in Chapter 4

Virus Name	GenBank Accession
A/black-headedgull/Netherlands/1/00(H13N8)	AY684886
A/black-headedgull/Sweden/1/99(H13N6)	AY684887
A/blackheadedgull/Astrakn/227/84(H13N6)	M26089
A/blackheadedgull/Astrakn/65/1983(H13N6)	EU580577
A/blackheadedgull/Mongolia/1756/2006 (H16N3)	GQ907294
A/blackheadedgull/Mongolia/1766/2006(H13N6)	GQ907302
A/blackheadedgull/Sweden/2/99(H16N3)	AY684888
A/blackheadedgull/Sweden/3/99(H16N3)	AY684889
A/blackheadedgull/Sweden/4/99/(H16N3)	AY684890
A/blackheadedgull/Sweden/5/99/(H16N3)	AY684891
A/blackheadedgull/Turkmenistan/13/76(H16N3)	EU293864
A/commongull/Norway/10_1617/2006(H16N3)	FM179755
A/daw/Germany/WV1141KR/03(H13)	AM087220
A/duck/Siberia/272/1998(H13N6)	AB284988
A/Fulicaatra/Volga/635/1986(H16N3)	EU564109
A/greatblackheadedgull/Astrakn/1420/79(H13N2)	EU293858
A/greatblackheadedgull/Astrakn/1421/79(H13N2)	EU293859
A/greatblackheadedgull/Astrakn/591/82(H13N2)	EU293860
A/greatblackheadedgull/Atyrau/2966/2008(H13N6)	GU953282
A/greatblackheadedgull/Atyrau/743/2004(H13N6)	GU982281
A/greatblackheadedgull/Atyrau/744/2004(H13N6)	GU982282
A/greatblackheadedgull/Atyrau/767/2004(H13N6)	GU982283
A/greatblackheadedgull/Atyrau/773/2004(H13N6)	GU982284
A/greatblackheadedgull/Gurjev/76/83(H13N2)	EU293861
A/gull/Astrakn/1314/1979(H13N2)	EU835898
A/gull/Astrakn/176/1986(H13N2)	EU835899
A/gull/Astrakn/1818/1998(H13N6)	EU835900
A/gull/Astrakn/1846/1998(H13N6)	EU580576
A/gull/Astrakn/226/1984(H13N6)	EU835895
A/gull/Astrakn/3483/2002(H13N6)	EU835897
A/gull/Astrakn/998/1990(H13N6)	EU835896

A/gull/Denmark/68110/2002(H16N3)	GQ247872
A/gull/Stralsund/Wv1136-40/03(H13N6)	AM922163
A/herringgull/Astrakn/458/85(H13N6)	EU293862
A/herringgull/Astrakn/479/85(H13N6)	EU293863
A/herringgull/Atyrau/2216/2007(H16N3)	GU953286
A/herringgull/Atyrau/280/2002(H13N6)	GU953278
A/herringgull/Germany/WV1136KRK/03(H13)	AM087221
A/herringgull/Norway/10_1623/2006(H16N3)	FM179756
A/herringgull/Norway/10_2336/2006(H13N6)	FM179758
A/kelpgull/Argentina/LDC4/2006(H13N9)	EU523136
A/Larusichthyaetus/Astrakn/10/1988(H13N6)	EU564106
A/Larusichthyaetus/Astrakn/44/1988(H13N6)	EU564115
A/Larusichthyaetus/Astrakn/75/1983(H13N2)	EU564107
A/Larusminutus/Astrakn/3357/2002(H13N6)	EU564108
A/Larusridibundus/Germany/R2064/2006(H13)	AM922164
A/littletern/Gurjev/779/83(H16N3)	EU148601
A/mallard/Gurjev/785/83(H16N3)	EU148600
A/Mongoliangull/Mongolia/401/2007(H13N6)	GQ907310
A/Mongoliangull/Mongolia/405/2007(H13N6)	GQ907318
A/slender-billedgull/Astrakhan/28/76(H16N3)	EU293865
A/teal/Volga/671/86(H16N3)	EU148602
A/yellow-leggedgull/Ukraine/912306/2005(H13)	EU599306
A/Americanwhitepelican/Minnesota/AI-07-1819/2007(H13N9)	CY054300
A/Americanwhitepelican/Minnesota/Sg-0611/2008(H13N9)	CY054302
A/black-leggedkittywake/Alaska/295/1975(H16N3)	CY015160
A/glaucous-winged gull/Southcentral	CY070850
Alaska/9JR0691R1/2009(H13N6)	This study
A/glaucous-winged gull/Southcentral	CY070858
Alaska/9JR0747R1/2009(H13N6)	This study
A/glaucous-winged gull/Southcentral	CY070866
Alaska/9JR0738R1/2009(H13N6)	This study
A/glaucous-winged gull/Southcentral	CY070874
Alaska/9JR0769R1/2009(H13N6)	This study
A/glaucous-winged gull/Southcentral	CY070882
Alaska/9JR0781R1/2009(H13N6)	This study

A/glaucous-winged gull/Southcentral	CY070890
Alaska/9JR0783R1/2009(H16N3)	This study
A/great black-backed gull/Newfoundland/296/2008(H13N2)	GU724153
	This study
A/gull/Maryland/704/1977(H13N6)	CY014694
A/gull/Massachusetts/26/1980(H13N6)	K00383
A/gull/Minnesota/945/1980(H13N6)	CY014720
A/herringgull/DE/475/1986(H13N2)	CY005914
A/herringgull/DE/712/1988(H16N3)	CY005933
A/herringgull/Delaware/660/1988(H13N6)	CY014603
A/herringgull/NJ/782/1986(H13N2)	CY005932
A/laughinggull/DE/2838/1987(H13N2)	CY005979
A/laughinggull/NewJersey/Sg-00485/2008(H13N9)	CY042429
A/laughinggull/NewJersey/Sg-00559/2008(H13N9)	CY042593
A/laughinggull/NewJersey/Sg-00568/2008(H13N9)	CY042607
A/ruddyturnstone/NewJersey/1407/2001(H13N6)	GQ117287
A/shorebird/DE/68/2004(H13N9)	CY005931
A/shorebird/Delaware/168/06/(H16N3)	EU030976
A/shorebird/Delaware/195/2006(H16N3)	CY045383
A/shorebird/Delaware/221/2006(H13N9)	CY043888
A/shorebird/Delaware/224/2006(H13N9)	CY043896
A/shorebird/NewJersey/840/1986(H16N3)	CY014599

Appendix 8

Availability of sequence information for AIV from gulls by segment (from GenBank and the Influenza Resource Database) (Chapter 4)

Region	Gene Segment[a]					
	PB2	PB1	PA	NP	M	NS
American	37	41	42	41	47	42
Eurasian (excluding H5N1)	16	15	14	23	20	19
Eurasian H5N1	11	11	10	11	11	10
Total	64	67	66	75	78	71

[a] Partial and complete sequences are included.

Appendix 9

Hemagglutinin and neuraminidase subtype combination frequency in viruses isolated from gull species globally (Chapter 4).

NA subtype	HA subtype															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1		1			20	1			1							
2						1	1		2				**9**			1
3	1	1			3		6						1			**11**
4						4				1	1	1				1
5									1				1	1		1
6			1	1							2		**37**			
7		2														
8		3	1		1	2	1						2			
9		3		1	1						1		**7**			
?						1							3			

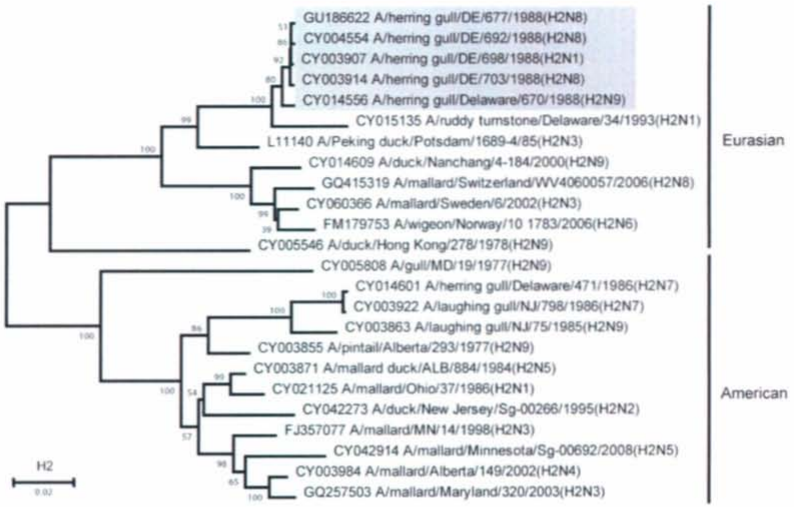
Blank cells denote the subtype has not been detected.

Values indicate the number of viruses isolated.

Values in bold denote the subtype combinations that occur most frequently, with the exception of H5N1

Appendix 10

Phylogenetic tree of H2 HA sequences from gulls H2 sequences



Phylogenetic tree of H2 HA sequences from gulls H2 sequences, in addition to reference sequences from other avian hosts. Viruses shaded in grey are those in clade 1 of Figure 4.3 and Table 4.3 (Chapter 4).