INFLUENZA-A VIRUSES IN DUCKS IN NORTHWESTERN MINNESOTA: FINE SCALE SPATIAL AND TEMPORAL VARIATION IN PREVALENCE AND SUBTYPE DIVERSITY

by

BENJAMIN ROBERT WILCOX

(Under the Direction of Michael J. Yabsley)

ABSTRACT

Waterfowl from northwestern Minnesota were sampled for Avian Influenza Virus (AIV) from July – October in 2007 and 2008. AIV was detected in 222/2,441 (9.1%) ducks in 2007 and in 438/2,452 (17.9%) ducks in 2008. AIV prevalence peaked in late summer. We detected 27 and 28 subtypes during 2007 and 2008, respectively. Ten hemagglutinin (HA) and all neuraminidase (NA) subtypes were detected each year. Subtype diversity varied between years and increased with prevalence. There were seven predominant subtypes detected during 2007 ($\geq$5% of subtype diversity), and six predominant subtypes detected during 2008. Within H2 and H12, H2N3 and H12N5 viruses predominated, respectively, but each were only detected during a single year. Mallards were the predominant species sampled, and 531 AIV were isolated from mallards (80.5% of total isolates). Juvenile mallard data from both years adequately described the temporal and spatial prevalence from the total sample and also adequately represented subtype diversity.

INDEX WORDS: Avian influenza, Subtype diversity, Waterfowl, Disease surveillance

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DEDICATION

This thesis is dedicated to my wife, Kristine, without which there would be no way that I could have completed this level of a project. Her dedication to our family, Wyatt and Kody, during my travels and study has been outstanding. I love you and thank you for all of the work that you have done so that I could complete my research.

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INTRODUCTION

Avian influenza virus (AIV) has become a disease of special concern within the last decade due to the outbreak of the highly pathogenic avian influenza (HPAI) virus strain, H5N1, in Asia [1]. This strain has been associated with numerous deaths of avian species [2] and people [3]. Although H5N1 HPAI viruses have not been detected in the United States, low pathogenic AIV strains are present in North American waterfowl populations. Currently, our understanding of the epidemiology of AIV in these duck populations is incomplete.

The reservoir for AIV is wild birds [2,4,5], especially birds in the Orders Anseriformes (ducks, geese, and swans) and Charadriiformes (shorebirds, gulls, and terns). Avian influenza viruses are transmitted via the fecal-oral route, and ducks, particularly those from the Anatini tribe (dabbling ducks), are infected annually [6]. North American birds in this tribe include the mallard (*Anas platyrhynchos*), blue-winged teal (*A. discors*), American green-winged teal (*A. crecca*), cinnamon teal (*A. cyanoptera*) American wigeon (*A. americana*), northern pintail (*A. acuta*), northern shoveler (*A. clypeata*), gadwall (*A. strepera*), American black duck (*A. rubripes*), mottled duck (*A. fulvigula*), and wood duck (*Aix sponsa*).

Life history traits of dabbling ducks play an important role in AIV transmission [7]. These species tend to feed in shallow freshwater marshes and lakes on various aquatic plants and invertebrates, and on seeds in crop fields. These traits coupled with a high density of birds at these locations provide and ideal situation for fecal oral transmission.

Pre-migration strategies of dabbling ducks during late summer and fall also influence transmission rates of AIV. Each year after nesting, ducks at northerly latitudes stage before migrating south to their wintering grounds. During this time, AIV prevalence peaks due to the presence of numerous naïve juvenile ducks concentrating at relatively few locations. Previous studies conducted in Minnesota reported that the prevalence of AIV shedding among mallards in September ranges from 6.7-11.6% [8,9]. High prevalence rates of AIV have also been found in waterfowl in Sweden from July through December, with peak prevalence detected in November [10]. In contrast, the prevalence of viral shedding among mallards in the southern United States (wintering areas) was typically <1-2% [4,11].

The goal of my study was to increase our understanding of the epidemiology of AIV transmission in northern Minnesota. To accomplish this, we sampled ducks from July – October in both 2007 and 2008. This period coincides with pre-migration staging and the migration of ducks to and from this area. The specific objectives of my study were to:

Objective 1: Describe within-season (mid-July to October) variation of AIV prevalence over a small geographic area.

> Hypothesis 1: Based on previous studies, I predict that there will be a peak in AIV prevalence in late summer or early fall.
>
> Hypothesis 2: I expect there will be some variation in the time of peak prevalence between years.

Objective 2: Determine the spatial, temporal, host, and interacting factors that influence prevalence in our study area.

> Hypothesis: I predict that these factors are not independent; thus a combination of factors including time of sampling, species sampled, and location will be what

drives AIV prevalence in northwestern Minnesota with time of sampling being the main factor.

Objective 3: Determine the effects of location, time, and host on the detection of subtype diversity.

Hypothesis: There may be numerous factors that contribute to the make-up of subtypes that are detected in northwestern Minnesota but time of sampling will be the most important factor in determining subtype diversity.

Objective 4: Evaluate sampling efforts necessary to obtain reliable surveillance data from a localized study.

Hypothesis: I predict that we may be able to reduce sampling efforts to 1 or 2 species and/or cohorts and still capture the same epidemic curve and subtype diversity as when all species and cohorts are sampled from July - October.

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CHAPTER 2

LITERATURE REVIEW

AVIAN INFLUENZA – DESCRIPTION

Avian influenza virus (AIV) is a member of the *Orthomyxoviridae* family, genus *Influenzavirus A.* It is a segmented negative-sense RNA virus [1]. AIV is composed of eight gene segments that encode 10 viral proteins including the surface proteins hemagglutinin and neuraminidase. AIV is extremely variable in shape ranging from spherical to filamentous [2].

Avian influenza viruses are classified by hemagglutinin (H) and neuraminidase (N) subtypes. These surface proteins are used because they serve as neutralization sites on the virus that have implications in both immunology and epidemiology (i.e., antigenic drift and shift). Influenza viruses have their own unique nomenclature consisting of the following parts:

1. Antigenic type (Influenza A, B, or C)
2. Species from which the virus was isolated
3. Location from which the isolate came (could be city, state, province, or county)
4. Laboratory or reference number for the isolate
5. Isolation year
6. The H and N subtypes

An example of this nomenclature is: A/Mallard/Minnesota/32434/2008 (H3N8).

Avian influenza virus can be either high pathogenic or low pathogenic. An AIV isolate is distinguished as being high pathogenic if the virus has an intravenous pathogenicity index (IVPI) >1.2 in 6-week old chickens. The IVPI is determined by inoculating 6-week old chickens with a suspect sample and monitoring them for 10 days [3]. The chickens are given a score (0-3) based on severity of disease, with 0 representing no disease and 3 representing death of the chicken. The mean score from observations on each of the 10 days is calculated and any samples with scores >1.2 are considered to be high pathogenic AIV. Alternatively, high pathogenic AIV are characterized as causing 75% mortality in 4, 8-week old chickens [4]. In addition, H5 and H7 viruses that do not meet the above criteria must be sequenced and compared to other H5 and H7 viruses to determine genetic similarity. All other viruses that do not meet any of the criteria stated are considered low pathogenic.

AVIAN INFLUENZA – HISTORY, HOST RANGE, AND RESERVOIR

Avian influenza virus was first isolated during a mortality event in South Africa in 1961 involving common terns (*Sterna hirundo*) [5]. The isolation of highly pathogenic H5N3 from these common terns was the only known outbreak of HPAI viruses from free-living wild birds until the more recent outbreak of the Asian strains of HPAI H5N1.

Over the next decade, antibodies to AIV were detected in numerous species of birds indicating that wild birds could potentially serve as the reservoir for AIV [6,7,8]. Subsequent virus isolations were also obtained from shearwaters in Australia [9] and ducks in California [10]. These findings not only supported the idea that many species of birds were exposed to AIV, but confirmed the hypothesis that wild birds could be the reservoir for AIV.

More than 100 species of wild birds in 12 avian orders have been exposed to AIV naturally. From 1970 to the present, publications have shown that AIV has a global distribution [11] with serologic evidence being found even in penguins in Antarctica [12,13,14,15]. All 16 H and 9 N subtypes have been isolated from wild birds [11,16,17].

Birds in the Orders Anseriformes (ducks, geese, and swans) and Charadriiformes (gulls, shorebirds, and terns) are the most common orders from which virus isolations are made [18]; thus, they are the accepted primary reservoirs for AIV. Within these orders, virus isolation rates vary between families and even species. In Anseriformes, the subfamily Anatinae (ducks) is the group from which most virus isolations are detected. In this subfamily, the mallard (*Anas platyrhynchos*) is the most represented species; however, the mallard is not the only species in this group that is important to the maintenance and transmission of AIV. Within the Order Charadriiformes, most AIV isolations are made from the families Laridae (gulls) and Scolopacidae (shorebirds). However, in Scolopacidae, most isolated viruses are from the ruddy turnstone (*Arenaria interpres*) [19]. Furthermore, most of the isolations from the ruddy turnstone come from one place, Delaware Bay, U.S., at one time of year, May through June [19].

The subtypes of viruses that are isolated in wild birds vary among orders and may divide the virus reservoir into different sections, with different groups of birds responsible for the maintenance of different subtypes [20,21]. Although a great diversity of AIV subtypes has been isolated from ducks, the H3, H4, and H6 subtypes are most common [16,22]. Furthermore, the diversity of virus subtypes found in ducks can vary greatly from year to year. In gulls, the predominant subtypes are H13 and H16. Other viruses commonly found in the Order Charadriiformes include H5, H7, and H9 viruses [21].

AVIAN INFLUENZA – TRANSMISSION AND MAINTENANCE

We need a greater understanding of the transmission and maintenance cycle of AIV before the complete cycle can be described accurately. Transmission between wild birds takes place via the fecal-oral route [23]. Replication of AIV occurs in the intestinal tract of ducks [24], and they shed large amounts of virus in their feces [25]. On average, muscovy ducks (*Cairina* *moschata*) shed approximately 1×10^{10} mean embryo infectious doses (EID_{50}) in 24 hours. Pekin ducks (domesticated subspecies of mallards) shed virus for more than 28 days [16]. This prolonged shedding of large amounts of virus into the environment increases the probability of virus being ingested by other waterfowl.

Because large amounts of virus are shed into the environment through feces and because most birds that are infected with AIV are associated with aquatic environments, the environment likely plays a role in the maintenance of AIV. Isolations of AIV have been made from surface water in numerous locations including Minnesota, Canada, and Alaska [16,26,27]. Sediment samples in Alaska have also tested positive by RT-PCR for AIV [28].

Data from laboratory trials have supported the hypothesis that environmental factors affect maintenance of AIV. The first study that looked at the potential of water to be involved in maintenance of AIV found that distilled water spiked with A/duck/Memphis/546/74 (H3N2) at a dose of $10^{8.1}$ EID_{50} was infective for 32 days [25]. Later work found that temperature, pH, and salinity affected the duration of AIV persistence in water [29,30]. There are few studies that have looked at effects of water chemistry, and it is possible that other factors could also affect AIV persistence such as bacteria, invertebrates, and metals. Also, different subtypes vary in their persistence rates when kept at uniform laboratory conditions [29,31]. More work is needed to better understand the role the natural environment truly plays in the maintenance of AIV.

Likely, a combination of environmental factors and bird-to-bird transmission are responsible for maintaining the virus within and between years [32]. Although numerous studies have shown that AIV transmission occurs on the breeding grounds [16,33,34], transmission on the wintering grounds has also been documented. Avian influenza virus was isolated from mottled ducks (*A. fulvigula*), a non-migratory resident bird of the southeastern United States

[22]. Between seasons, bird-to-bird transmission likely facilitates maintenance of the virus from the breeding grounds to the wintering grounds and back to the breeding grounds the following year. The role of the environment is less clear but it may serve as a medium for virus persistence between times when ducks are absent on lakes.

Other factors, such as the role of individual species, population density, behavior, feeding habits, and susceptibility have on the maintenance cycle of AIV, also need to be better understood [22,35,36]. With migration habits of different species being highly variable, there may be more complicated aspects to take into account to fully understand the true maintenance cycle of AIV.

NORTH AMERICAN WATERFOWL – AN IN-DEPTH LOOK

The Order Anseriformes is composed of ducks, geese, and swans. There is great diversity in isolation rates of AIV within this order. In fact, most AIV isolations come from the dabbling ducks (tribe Anatini) [11,17,18]. Within this tribe, most of the isolations come from mallards, but numerous isolates have been obtained from blue-winged teal (*A. discors*) and northern pintail (*A. acuta*) [11,17.34,35,37]. Other species in this group include the American wigeon (*A. americana*), American black duck (*A. rubripes*), green-winged teal (*A. crecca*), cinnamon teal (*A. cyanoptera*), northern shoveler (*A. clypeata*), mottled duck, and wood duck (*A. sponsa*); however, isolation rates for AIV from these species vary depending on location and time of year [17,37].

Geese, though numerous and inhabiting most of North America, are not viewed as being tremendously important in the transmission of AIV. Though there are some reports of virus isolations from wild geese [38,39], they are not common [37,40,41,42]. A study conducted in Canada found that Canada geese (*Branta canadensis*) shed virus for ≤3 days [43]. There have also been reports of seroconversion within Canada geese [6,42,44] that would imply they are exposed to AIV while sharing similar habitat with other waterfowl species. Laboratory trials looking at duration of viable virus in goose feces and in water contaminated with goose feces also showed that the length of persistence of the virus is low compared to the estimates for ducks [44].

There are general spatial and temporal trends that AIV isolation rates follow in North America. AIV prevalence rates peak, sometimes as high as 30%, during late summer and early fall [37]. The peak corresponds to susceptible juvenile birds concentrating during pre-migration staging. The virus-isolation-based prevalence rates from northern breeding areas is much greater than those detected on southern wintering grounds where prevalence rates are typically lower than 2% [22,45]. During migration, prevalence falls off fairly rapidly as the birds travel south.

The following is a review of various studies throughout North America.

Early studies of AIV in Waterfowl

The first indication that waterfowl may be a potential reservoir of AIV was from a study conducted in the early 1970s from California [10]. Forty-eight AIVs were isolated from more than 2000 wild and domestic ducks from October to December, 1972. In addition, a high diversity of subtypes was detected in this study suggesting genetic recombination could occur.

In September 1973, tracheal swabs were taken from 80 mallards and wood ducks at Roseau Wildlife Refuge in northwest Minnesota [33]. Four of the birds sampled tested positive for AIV. Subtyping of these isolates was not conducted.

In a study of AIV in hunter-killed waterfowl from Maryland and Delaware, numerous species were sampled in November and 4 AIV isolations were made from 159 birds sampled

(3%) [38]. Species from which virus was isolated include 1 mallard, 2 American black ducks, and 1 Canada goose.

One of the first studies of AIV in Canada focused on waterfowl over a 3-year period, 1976-1978, in Alberta, Canada [16]. AIV was isolated from 1262 of 4827 (26%) ducks, showing that northern breeding populations were exposed at high rates. This study was the first to associate the large numbers of birds gathering in areas before migration with increased AIV transmission. Mallard and northern pintail had the highest prevalence rates, 29% and 34%, respectively. Blue-winged teal and American wigeon followed with rates in the mid to upper teens. During this study, large numbers of the subtypes H4N6 and H6N2 were found but overall, distribution of subtypes was not restrictive to individual species. Throughout the 3-year study it was also determined that the predominant strains of AIV circulating in waterfowl change every year.

AIV Studies - Late 1970s to the Present

A continuation of the previous work in Canada [16] examined waterfowl in a comparison between Canada and New York during the month of August [37,40]. In Alberta, Canada, 9195 birds were sampled and AIV was isolated from 2425 of them (26%) compared to 168 AIV isolates from 1430 samples taken (12%) in New York. There were tremendous differences in prevalence among species: mallards (42%), American black ducks (30%), blue-winged teal (11%), wood ducks (2%), and Canada geese (0%) [40]. They also were able to classify 44 different subtype combinations including hemagglutinin types 1-12 and neuraminidase types 1-9. The most frequent combinations were H3N8, H4N6, and H6N2 [37]. This study was also the first to find that juvenile birds had higher prevalence rates (30%) than adult birds (11%) [37].

Another pair of studies looked at AIV in the early 1980s after an outbreak of highly pathogenic H5N2 AIV occurred in domestic poultry in Pennsylvania, New Jersey, Maryland, and Virginia from November 1983 to May 1984. In the first study, a task force was assembled to test birds in the areas where the outbreak occurred [39]. This study was designed to detect highly pathogenic H5N2 in wild birds; however, only one bird was found with the H5N2 virus, a pen-raised chukar (*Alectoris chukar*) in Pennsylvania. A follow-up study was conducted in the same area examining waterfowl from June-November 1984 [46]. Researchers obtained 171 AIV isolates (8%) from 2043 wild birds (primarily waterfowl) tested. Numerous subtypes were found but the dominant virus was H6N2; however, relatively large numbers of H3N8 and H4N6 were also detected. An H5N2 virus was detected during this study, but sequence analysis clearly distinguished it from the highly pathogenic virus that infected poultry the year before. During this time frame, Smitka and Maassab [47] were studying AIV in Michigan and they detected one AIV isolate from a mallard out of 100 sampled waterfowl.

In the late 1980s and early 1990s, surveillance studies continued to show that viruses were detected in waterfowl throughout the breeding range. In Ohio, a 3-year study was implemented to determine if AIV could be isolated from waterfowl in the state and determine if their domestic turkey flocks were at risk of exposure [48]. Of 928 waterfowl samples collected during October and November, 55 (6%) were AIV virus isolation positive. Numerous subtypes were detected during this study with the most common being those of the H3 and H11 hemagglutinin groups.

In the early 1990s, AIV was studied on waterfowl wintering grounds in Louisiana [22]. AIV was isolated from numerous species of waterfowl including resident mottled ducks, indicating transmission does occur on the wintering grounds. However, prevalence was low, with AIV isolated from only 28 of 1389 (2%) ducks. Numerous subtypes were isolated from waterfowl; however, H4 was the most common hemagglutinin type found and H4N6 the most common combination detected.

From 1998-2000, Hanson et al. [34] sampled mallards and northern pintails during the month of September in Marshall and Roseau Counties, Minnesota. Mean prevalence of AIV isolated from these birds during the 3-year study was 10.8% (154 positive isolations of 1423 sampled ducks) and most isolations were from juvenile birds. In fact, prevalence in juvenile birds exceeded 20% in mallards both in1999 and 2000. Many subtypes were also isolated during this time but the H3, H4, and H6 hemagglutinin subtypes showed up most frequently. Common hemagglutinin and neuraminidase combinations during the three years included H3N8, H4N6, H6N8, H9N2, and H11N9. There were also numerous mixed infections detected.

Another study examining AIV on winter sites was conducted in Brazoria County, Texas in 2001 and 2002 [49]. During this study, a high prevalence rate (>10%) relative to earlier work in Louisiana [22] was detected. Another interesting discovery in the study by Hanson et al. [49] was the lack of common subtypes generally associated with waterfowl (H3, H4, and H6). Subtypes classified included H2N4, H7N3, H7N4, and H8N4 in 2001, and H1N4, H1N3, H2N4, H2N9, H7N3, and H7N4 in 2002. These subtypes typically account for <1% of those isolated on breeding grounds.

From 2004-2006, wintering area isolation data were collected in North Carolina from hunter-killed ducks [45]. AIV was isolated from 23 of 2904 (<1%) birds sampled over this 3-year period. Most of the isolated AIV were from ducks of the Anatini tribe, but two bufflehead (*Bucephala albeola*) (trive Mergini) were positive. Subtypes were highly variable but H3N8, H4N6, H5N1/H4N1, H10N7, and H7N3 were detected most commonly.

Recently, due to concerns of HPAI H5N1 being introduced to North America from Asia, surveillance efforts in Alaska have intensified. A study by Ito et al. [27] in Alaska conducted during the breeding season found that the prevalence of AIV was low (4%); only 108 of 3120 waterfowl tested positive. From May 2006 through March 2007, 1.5% of wild birds were positive for AIVs, determined by virus isolation [50]. Of these birds, 7% of surface feeding ducks were positive (no specific numbers given). Therefore, though overall prevalence was low, the greater prevalence in surface feeding ducks is consistent with the higher prevalence rates seen in other waterfowl studies.

LOW PATHOGENIC AVIAN INFLUENZA – GLOBALLY

AIV has a global distribution [11]. Many surveillance studies have been conducted, and other studies have examined some of the same types of spatial and temporal analyses that have been studied in North America. Presented here are some of the more recent studies that were conducted outside of North America.

From January through March 1997, mallards were sampled in New Zealand as part of a study that examined both virus isolation and serology [51]. Six AIV were detected from 321 mallards (1.9%). However, 109 sera samples were positive from the 335 (32.5%) total samples tested. Subtypes detected in this study were two H5N2 viruses and four H4N6 viruses.

In 1998, aquatic birds were tested for AIV around ostrich (*Struthio camelus*) farms in South Africa from May to July, just before the time when most AIV outbreaks occur in the ostriches [52]. Samples were taken from 262 aquatic birds and isolates were found in 8 of the samples (3%), all of which were H10N9.

Two AIV isolations were obtained from pooled samples of graylag geese (*Anser anser*) and whooper swans (*Cygnus cygnus)* in Northern Ireland in 1997 [53]. Both samples were

H6N1. Because samples were pooled, there is no way to give an accurate prevalence estimate. A 4-year surveillance study took place at various locations throughout Ireland from 2003-2007 [54]. A total of 3341 hunter-killed waterfowl was sampled during the winter months. Swabs were pooled according to species with no more than five samples per media tube. From these pools, 32 AIVs were isolated from one location, the Wexford Sloblands, in southeast Ireland. Numerous subtypes were found throughout the study including H10N7 and H6N2; however, no H3 or H4 subtypes were recovered.

From 2002-2005, a large scale project was conducted at Ottenby Bird Observatory, located on a small island in the Baltic Sea [55]. This study involved surveillance and it included temporal analysis of AIV. This study looked at 4,800 waterfowl of 16 species. Wallensten et al. [55] used PCR to determine that 600 of the sampled waterfowl tested positive for AIV (12.5%), and all but 25 were from mallards. Samples were taken from March through December in most years. Fall prevalence (July-December, 3-26%) was higher than spring prevalence (March-June, 0-10%). In the fall, the prevalence in juveniles was 20% and 12% in adults. These prevalence rates are consistent with data from North America. Forty subtype combinations were detected during this study but the most common ones were H4N6, H7N7, and H6N2.

AIV has never been isolated from birds in Antarctica but serologic evidence of exposure has been documented in various penguin species [12,13,14]. In a study by Wallensten et al. [15], swabs from Adélie penguins (*Pygoscelis adeliae*), chinstrap penguins (*Pygoscelis antartica*), and gentoo penguins (*Pygoscelis papua*) were uniformly negative for AIV, but 9 of 76 (12%) gentoo penguins were serologically positive.

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CHAPTER 3

INFLUENZA-A VIRUSES IN DUCKS IN NORTHWESTERN MINNESOTA: FINE SCALE SPATIAL AND TEMPORAL VARIATION IN PREVALENCE AND SUBTYPE DIVERSITY[1]

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ABSTRACT

Waterfowl from northwestern Minnesota were sampled by cloacal swabbing for Avian Influenza Virus (AIV) from July – October in 2007 and 2008. AIV was detected in 222 (9.1%) of 2,441 ducks in 2007 and in 438 (17.9%) of 2,452 ducks in 2008. Prevalence of AIV peaked in late summer. We detected 27 AIV subtypes during 2007 and 31 during 2008. Ten hemagglutinin (HA) subtypes were detected each year (i.e., H1, 3-8, and 10-12 during 2007; H1-8, 10 and 11 during 2008). All neuraminidase (NA) subtypes were detected during each year of the study. Subtype diversity varied between years and increased with prevalence into September. Predominant subtypes during 2007 (comprising $\geq$5% of subtype diversity) included H1N1, H3N6, H3N8, H4N6, H7N3, H10N7, and H11N9. Predominant subtypes during 2008 included H3N6, H3N8, H4N6, H4N8, H6N1, and H10N7. Additionally, within each HA subtype, the same predominant HA/NA subtype combinations were detected each year and included H1N1, H3N8, H4N6, H5N2, H6N1, H7N3, H8N4, H10N7, and H11N9. The H2N3 and H12N5 viruses also predominated within the H2 and H12 subtypes, respectively, but only were detected during a single year (H2 and H12 viruses were not detected during 2007 and 2008, respectively). Mallards were the predominant species sampled (63.7% of the total), and 531 AIV were isolated from this species (80.5% of the total isolates). Mallard data collected during both years adequately described the observed temporal and spatial prevalence from the total sample and also adequately represented subtype diversity. Juvenile mallards also were adequate in describing the temporal and spatial prevalence of AIV as well as subtype diversity.

Key words: avian influenza, subtype diversity, waterfowl, disease surveillance

INTRODUCTION

Wild birds of the order Anseriformes (ducks, geese, and swans) are important reservoirs for avian influenza virus (AIV) [1,2]. Within these populations, transmission occurs through a fecal-oral route [3]. Prevalence of AIV in ducks varies by species, age, time, and location [2]. Most AIV isolations from North America and Europe have been reported from mallards (*Anas platyrhynchos*) and other species in the subfamily Anatinae, tribe Anatini (i.e., dabbling ducks) [2,4-6]. Juveniles tend to have greater infection rates than adults [1], probably because of the immunologically-naïve status of the former age cohort [4]. In North America, prevalence rates of AIV tend to peak during late summer, when waterfowl aggregate prior to fall migration [1]. Prevalence of infection at that time can be as high as 30% [1]. In contrast, prevalence of AIV infection in ducks on wintering grounds in the southern United States generally is $\leq$2% [7].

All 16 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes of AIV exist in wild bird populations. However, the H1-12 subtypes predominate in wild duck populations in North America; other subtypes are either associated with gulls (H13, H16) or have not been reported from North America (H14, H15) [1,5,6,8,9]. Within Anseriform populations in both North America and Europe, the H3, H4, and H6 subtypes consistently account for most of the subtype diversity, and certain HA/NA combinations (e.g. H3N8, H4N6) appear to be over represented in these reported isolates [1,6,7,10,11].

There have been numerous studies related to the natural history of AIV in wild ducks [5]. Currently, there are global efforts to monitor AIV prevalence in such populations [6,12]. Although these efforts clearly have defined general spatial, temporal, and species-related epidemiologic patterns at a continental scale, few researchers have examined these relationships on a finer scale. Information related to short-term prevalence or subtype diversity related to fine-scale spatial and temporal variation in sampling efforts not only has application to understanding the epidemiology of these viruses in duck populations, but also the design and implementation of effective and cost-efficient surveillance programs.

In this study, we examined AIV in duck populations in Minnesota, USA from mid-summer to early fall during 2007-2008. The study period corresponds with the pre-migration congregation (hereafter, staging) and southward migration from this geographic area. To provide a historical perspective, we selected study sites at which avian influenza previously had been investigated. At Roseau River Wildlife Management Area (WMA), 7% of 60 mallards sampled during September 1973 were positive for AIV [13]. Similarly, 10.8% of 1,423 mallards and northern pintails (*Anas acuta*) sampled from Marshall and Roseau Counties, Minnesota during September 1998-2000 were positive for AIV [14]. The goals of the current study were to: 1) describe within-season (mid-July to October) variation of AIV prevalence over a small geographic area; 2) determine the spatial, temporal, host, and interacting factors that influence prevalence in our study area; 3) determine the effects of location, time, and host on the detection of subtype diversity; and 4) evaluate sampling efforts necessary to obtain reliable surveillance data from a localized study.

METHODS

Ethics Statement

All procedures involving animals were approved by the University of Georgia Institutional Animal Care and Use Committee (A2010 6-101). Animals were collected under Minnesota Division of Fish and Wildlife permit 16282 and U.S. Fish and Wildlife Service, U.S. Department of the Interior permit MB779283-0.

Study Sites

All study sites were located in Northwestern Minnesota. The study sites were the Bemidji area (Beltrami County - 47°28’25.03”N, 94°52’49.00”W), Fosston area (Polk County - 47°34’36.31”N, 95°45’15.31”W), Thief Lake WMA (Marshall County - 48°29’12.85”N, 95°57’02.17”W), Roseau River WMA (Roseau County - 48°58’39.77”N, 96°00’32.08”W), and Agassiz National Wildlife Refuge (NWR; Marshall County - 48°18’02.90”N, 95°58’49.68”W; Fig. 1).

Sample Collection

Ducks were captured from July to October of 2007 and 2008 using 3 techniques that were dependent on the time of year: drive trapping, night lighting, and rocket-netting. We also sampled hunter-harvested birds during waterfowl hunting season. Birds were classified as juvenile or adult based on plumage characteristics [15], and AIV samples were collected as previously described [14]. Specifically, cloacal swabs were obtained using sterile cotton-tipped applicators (Puritan® Medical Products Company LLC, Guilford, ME) and placed in 2 ml of Brain Heart Infusion media (Becton Dickinson and Co., Sparks, MD) supplemented with penicillin G (1,000 units/ml), streptomycin (1mg/ml), kanamycin (0.5mg/ml), gentamicin (0.25mg/ml), and amphotericin B (0.025 mg/ml) (Sigma Chemical Company, St. Louis, MO). Samples were stored at 4C (24-72hrs), shipped overnight, and frozen at -80C until processed.

Virus Isolation and Subtyping

To isolate viruses, samples were thawed, vortexed for 15s, and centrifuged at 1,500 x *g* for 15 min. The supernatant was inoculated (0.25 ml/egg) into four 9-11 day old specific-pathogen-free (SPF) embryonated chicken eggs via the allantoic route [7]. Eggs were incubated at 37C for 120 hr, after which amnio allantoic fluid was collected and tested by hemagglutination

(HA) assay [16]. All HA-positive samples were tested by AIV matrix RT-PCR using primers from Fouchier (2000) [17]. Subtyping was done at the National Veterinary Services Laboratories (NVSL), Ames, Iowa using hemagglutination inhibition and neuraminidase inhibition tests [18] and via genotyping of the HA and NA genes at the University of Minnesota, St. Paul, Minnesota [19, 20] and the University of California Davis [21] using standard Sanger sequencing for HA and NA segments. BLAST non-redundant database searching algorithm was applied to assign specific subtypes.

Data Analysis

Univariate associations between predictor variables and the results of virus isolation testing were evaluated using a chi-square test of independence. Multivariable analysis was performed using logistic regression with virus isolation as the dependent variable. Robust standard errors were used to account for the lack of independence between birds captured from the same site during a sampling season. Variables having a univariate association ($P < 0.2$) with influenza prevalence were eligible for inclusion in the multivariable analysis. Variables were removed from the multivariable model in a stepwise fashion based on their level of significance until only those with $P < 0.1$ remained. After reaching a preliminary main-effects model, all possible two-way interactions were evaluated. Date of sample collection was evaluated as both a categorical variable (i.e., month) and as a continuous variable (i.e., week and day), with Akaike's Information Criterion (AIC) being used to determine its most appropriate functional form as a predictor. The fit of the final model was evaluated using the Hosmer-Lemeshow goodness of fit test. Residuals and influence statistics were used to screen for influential covariate patterns by plotting the delta deviance, delta chi-square, and delta beta values versus predicted probabilities. All analyses were performed using commercially available statistical software (Stata version

11.0, StataCorp LP, College Station, TX). Hypothesis tests assumed a two-sided alternative hypothesis and P-values < 0.05 were considered statistically significant.

RESULTS

During 2007 (July 11 – October 28) and 2008 (July 10 – October 15), 2,441 and 2,452 ducks were sampled in Minnesota, respectively (Table 1). Avian influenza viruses were isolated from 222 (9.1%) of sampled ducks during 2007 and from 438 (17.9%) of birds during 2008. Based on chi-square test of independence, the prevalence of AIV differed between mallards, other dabbling ducks (Tribe: Anatini), and the combined sample of divers (Tribes: Athyini, Mergini, Oxyurini) and wood ducks (*Aix sponsa*; S1 Table). Peak prevalences in mallards during 2007 and 2008 were approximately 20% and 35%, respectively.

During both 2007 and 2008 AIV prevalence peaked in late summer (late August or early September; Fig. 2). Based on chi-square testing of independence, prevalence significantly differed between years, month of sampling, age, and location (S1 Table). Because the characteristics of sampled birds varied over time and location, a multivariable logistic regression analysis was performed to evaluate potential interactions and obtain adjusted effect estimates. The low prevalence of AIV in both the Fosston and Bemidji locations resulted in computational problems when interactions between location and the other variables were evaluated, and consequently observations from Fosston and Bemidji (n = 330) were excluded from the multivariable analysis. Because the preliminary analysis identified interactions between year, sampling date, and location; intermediate model selection steps were performed separately for each year. Ultimately, the same combination of predictors was identified as the best fitting model based on AIC comparisons for both 2007 and 2008. Both of these year-specific models (not reported) included an interaction between location and sampling date. The best fit of both models occurred when sampling date was included as the week of sample collection in its quadratic form (i.e., week and week^2). The sampling week variable was centered by subtracting the mean (week 36) from each observation to prevent collinearity with the squared week term. Because models for both years shared the same predictors, data from 2007 and 2008 were combined in the final multivariable model, which contained a significant three-way interaction between location, week of sample collection, and year. All interactions and main effects in the final model were also statistically significant, and results of the Hosmer-Lemeshow test indicated that the model fit the data well (H-L goodness-of-fit test, $P = 0.84$).

Results of the final multivariable model are reported in Table 2. Adjusted effect estimates for the age and species type variables in this model indicated that the odds of having a positive virus isolation result were 2.7 times higher for juvenile birds than for adults, and compared to mallards, odds ratios were 0.10 for divers and wood ducks, and 0.32 for the other dabblers. Overall, subtypes were determined for 607 of the 660 (93.5%) AIV isolated. During 2007, 27 subtypes were detected in 190 AIV isolates, and 31 subtypes were detected in 417 AIV isolates during 2008 (Fig. 3). Predominant subtypes during 2007 (representing at least 5% of subtype diversity) were H1N1, H3N6, H3N8, H4N6, H7N3, H10N7, and H11N9. Predominant subtypes during 2008 were H3N6, H3N8, H4N6, H4N8, H6N1, and H10N7. Overall, 10 HA subtypes were detected each year: H1, 3-8, and H10-12 during 2007 and H1-8, 10 and 11 during 2008. All NA subtypes were represented each year.

Although subtype diversity varied between years (Fig. 3), similar patterns were observed during each year. During both years, the diversity of subtype combinations increased with the total number of isolates (S2 Figure). During 2007, six subtypes were detected in viruses isolated during August; this increased to 23 subtype combinations isolated during early September and decreased to 11 subtypes detected during late September. During 2008, 13 subtypes were represented in July/August isolations, 27 subtypes in early September isolates, and 11 were detected during late September/early October. An overlap between predominant subtype combinations was observed between years with the H3N6, H3N8, H4N6, and H10N7 viruses, each comprising more than 5% of total isolates each year (Table 3). Additionally, within each HA subtype, the same predominant HA/NA subtype combinations were detected each year and included H1N1, H3N8, H4N6, H5N2, H6N1, H7N3, H8N4, H10N7, and H11N9. The H2N3 and H12N5 viruses also predominated within the H2 and H12 subtypes, respectively, but were only represented in a single year (H2 and H12 viruses were not detected during 2007 and 2008, respectively).

Subtype diversity detected at the three northwestern study sites (Thief Lake WMA, Roseau River WMA, Agassiz NWR) is reported in Table 4. Only subtypes represented by more than one isolate during 2007 or 2008 are included and overall these included 13 and 20 subtypes during those years, respectively (Table 4). For the combined years, 25 of the 33 subtypes (76%) were detected at more than one of the study sites. The probability of detection of a specific subtype at multiple locations increased with the number of isolates of that subtype (Fig. 4).

Mallards were the predominant species sampled (63.7% of the total sample) and were the species with the greatest prevalence of AIV (80.5% of total detections). To determine whether surveillance of this species would adequately describe temporal variations of prevalence and subtype diversity at the northwestern Minnesota study sites, we compared annual data derived from mallards and from the total sample (i.e., all species). The mallard data in both years adequately described temporal prevalence (Fig 2) and subtype diversity (S3 Table). With regard to subtype diversity, only one subtype combination detected in the 2007 total sample and four

2008 subtype combinations were not represented in mallards during those years. These were an H6N6 detected in a northern pintail sampled during 2007 and an H3N9 from a green-winged teal, H4N5 from a green-winged teal, H10N6 from a blue-winged teal, and H10N8 from an American wigeon that were isolated during 2008. Juvenile mallards comprised 39.6% of the total sample. Further, all but four detected subtypes (H1N4, H6N6, H7N8, and H8N4) were represented in juvenile mallards in 2007, and only five of the total subtypes (H3N9, H4N5, H10N6, H10N8 and H11N9) were not isolated from juvenile mallards detected from 2008 samples. Overall, the temporal pattern of AIV in juvenile mallards (Fig. 2D) was similar to that observed for the total duck sample (Fig. 2A) and the entire mallard sample (Fig. 2B) during both years.

DISCUSSION

Our results indicate a seasonal trend in AIV prevalence within northwestern Minnesota that is consistent with previously described seasonal patterns in North America. This trend consists of a seasonal peak that starts in late July and peaks during August [2,4]. Locally, our observations also are consistent with a previous study in Minnesota from 1980-1983 that used a sentinel mallard system. Specifically, infected mallards in the earlier study first were detected during late July and early August during all four years of that study [22]. Prevalence and the specific timing of this peak varied slightly between years (Fig 2). Further, our predictive multivariable model suggests that temporal patterns may not be synchronized among study sites (S2 Figure). Variations in seasonality were apparent in previous studies conducted in North America [23] and Europe [12]. In New York, prevalence peaked in late September [23], and in Europe, the peak appeared to extend from September until December [12[] These differences may be influenced by variations of migratory timing, population structure, or species abundance and diversity at different sites.

Consistent with previous studies [2,4,6], AIV prevalence was dependent on species and age. Mallards were much more likely to be infected than the combined samples of dabbling ducks or the diving ducks and wood ducks. Overall, prevalence in the dabbling duck species included in this study ranged from 7% in American wigeon (*Anas americana*) to 37% in northern shoveler. These species-specific differences may be attributed to population density (mallards), behavior (dabbling ducks and wood ducks), feeding habits (surface feeding) [24], or possibly differences in susceptibility (diving ducks and wood ducks). Mallards have been suggested as the most important duck species related to AIV natural history, but the potential roles of other species in the maintenance and long-range movement of AIV should not be discounted. For example, a high prevalence of AIV infection in northern shoveler (*Anas clypeata*) has been reported from birds sampled on waterfowl wintering grounds [2]. Likewise, both blue-winged teal (*Anas discors*) and northern pintail have been suggested in the long-distance movement of these viruses [7,25].

Juveniles were almost three times more likely to be infected with AIV than adults (Table 2). This relationship has been previously reported [4] and has been observed in numerous studies worldwide [5,6,12]. This may be related to pre-migration staging and the resultant concentration of immunologically naïve juveniles [4].

Because our sampling strategy identified interactive effects between location and time (2 week sampling periods) (Table 2), the marginal effects of location on AIV prevalence could not be independently evaluated. Because environmental and habitat-related characteristics of a wetland (e.g., water depth, water temperature and pH, or avian species composition utilizing the habitat) potentially could influence AIV transmission rates [26], additional work is needed to fully understand this potentially important variable.

Subtype diversity varied over the study period during both years, but most North American HA and NA subtypes were detected during both 2007 and 2008 (Fig. 3). Subtype diversity reflected the observed AIV prevalence (Fig. 2). Few virus subtypes were found early (early July) in either field season, but the number of subtypes increased from August into September and decreased in late September and October. The increased subtype diversity over time may reflect increased detectability related to peak prevalence, the introduction of new subtypes related to migration into the study sites, or reassortment events. In relation to detectability, subtype-specific prevalence (S3 Table) often is very low. Specifically, the prevalence of an individual subtype (all species) ranged from 0.04-1.88% and 0.04-4.65% during 2007 and 2008, respectively. Two observations about migration were consistent between years. During both years, several predominant subtypes detected in July and August persisted to late September /early October (H3N8, H4N6, and H10N7 during 2007; H3N8 and H4N6 during 2008). Additionally, several subtypes (H7N3, H11N9, H1N1 during 2007 and H6N1 during 2008) were not detected until early September, when migrating birds probably began arriving in northwestern Minnesota.

The significance of reassortment to the natural history of AIV in wild bird populations is not well defined. The diversity observed within the H3 and H4 subtypes during 2007 and 2008 suggests that reassortment is common. During both years, H3N8 and H4N6 were the predominant subtypes. Numerous NA subtypes were associated with these HA types (H3N1, H3N3, H3N6, H4N8 during 2007; H3N1, H3N2, H3N3, H3N4, H3N6, H3N7, H3N9, H4N2, H4N4, H4N5, H4N8 during 2008). Similar diversity was associated with H6, H10, and H11 viruses during 2007 and H6 and H10 viruses during 2008 (S3 Table). In contrast, only one subtype combination was observed with the H8 subtype (H8N4): this cannot be explained but is consistent with other studies (S3 Table). Reassortment events are dependent on AIV coinfections, which have been reported in wild duck populations [10]. Because it is intuitive that the likelihood of coinfection is dependent on the prevalence of the potential parent viruses, the relationship between subtype diversity within an HA subtype and the number of isolates recovered is predictable (Fig. 4).

Although the subtypes detected varied annually, most HAs were detected each year. The dominant HA subtypes reported in North American waterfowl are H3, H4, and H6 viruses [2], which may cycle in these populations because of temporal changes in flock immunity [11]. Other HA subtypes have been reported as underrepresented (H2, H5, H7, H8, H9, and H13) or occur sporadically in ducks (H1, H10, H11, and H12; 11). In our study, HA diversity was consistent with this pattern. The H4 viruses were most common during 2007, the H3 viruses were most common during 2008, and the H6 viruses were well represented during 2008. With the exception of H2, H9 and H12, all other HA subtypes were represented both years but prevalence often was very low (S3 Table). The detection of most of these subtypes each year appears to reflect sampling intensity and the high number of isolates recovered. This observation implies that almost all of these HA subtypes are present at the northwestern Minnesota study sites annually.

During both 2007 and 2008, some subtype combinations were over represented. The predominant subtype combinations within the HA types were H1N1, H2N3, H3N8, H4N6, H5N2, H6N1, H7N3, H8N4, H10N7, H11N9, and H12N5. This was consistent between years.

Interestingly, with the exception of H6N1 and H7N3 (H7N7 in Europe) [6], these are the same subtype combinations that have been most reported from ducks in long-term studies in North America [2,4,23] and Europe [6,12] (Table 3). It is unknown why these predominant subtypes have been consistently detected in North America since 1976.

Our results suggest that AIV surveillance in northwestern Minnesota could be efficiently achieved by limiting testing to juvenile mallards (Fig. 2; S3Table). Prevalence and subtype diversity could be adequately described with a much reduced sample size based on juvenile mallards alone: in 2007, juvenile mallards represented 35.7 % (871/2441) of the total sample and in 2008 juvenile mallards represented 47.3% (1,065/2252) of the total ducks sampled. These results likely were influenced by the large proportion of mallards (S1 Table) in our total sample, but this approach should be considered when testing avian communities that are dominated by this species. Such an approach has application to efficiently recovering AIVs from the field and detecting annual variations in prevalence. This approach may not be applicable to situations in which the ecology of these viruses is less understood, where study questions relate the role of individual species in the natural history of these viruses, where mallards are not the predominant species, or in studies on the wintering grounds of North American waterfowl. Additionally, some subtypes such as the H9, H13, and H16 viruses are underrepresented or absent in ducks and may not be detected by this approach. Finally, although subtype diversity was captured, additional work is needed to determine if genetic diversity was adequately represented in the viruses recovered.

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Table 3.1. The prevalence of AIV in waterfowl by species and year from northwestern Minnesota, USA.

Species[a]	2007		2008		Total	
	N	No. Pos (%)	N	No. Pos (%)	N	No. Pos (%)
Mallard (*Anas platyrhynchos)*	1691	185 (11.0%)	1426	346 (24.3%)	3117	532 (17.1%)
Other Dabblers						
Gadwall (*Anas strepera*)	12	1 (8.3%)	13	1 (7.7%)	25	2 (8.0%)
American Wigeon (*Anas americana*)	10	1 (10.0%)	52	3 (5.8%)	62	4 (6.5%)
American Black Duck (*Anas rubripes)*	8	1 (12.5%)	1	0 (0%)	9	1 (11.1%)
Blue-winged Teal (*Anas discors*)	193	21 (10.9%)	468	31 (6.6%)	661	52 (7.9%)
Northern Shoveler (*Anas clypeata*)	10	0 (0%)	61	26 (42.6%)	71	26 (36.6%)
Northern Pintail (*Anas acuta*)	31	2 (6.5%)	47	6 (12.8%)	78	8 (10.3%)
Green-winged Teal (*Anas crecca*)	116	5 (4.3%)	191	23 (12.0%)	307	28 (9.1%)
Wood Ducks and Divers						
Wood Duck (*Aix sponsa*)	186	0 (0%)	20	1 (5%)	206	1 (0.5%)
Redhead (*Aythya americana*)	3	1 (33.3%)	12	0 (0%)	15	1 (6.7%)
Ring-necked Duck (*Aythya collaris*)	83	4 (4.8%)	138	1 (0.7%)	221	5 (2.3%)

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[a] Also includes Mallard/Gadwall Hybrid (n=1, 2007, *Anas platyrhynchos/strepera*); Mallard/American Black Duck Hybrid (n=5, 2007; 1, 2008, *Anas platyrhynchos/rubripes*); Canvasback (n=18, 2007; 1, 2008, *Aythya valisineria*); Greater Scaup (n=2, 2007, *Aythya marila*); Lesser Scaup (n=23, 2007; 11, 2008, *Aythya affinis*); Bufflehead (n=5, 2007; 3, 2008, *Bucephala albeola*), Common Goldeneye (n=24, 2007; 6, 2008, *Bucephala clangula*); Hooded Merganser (n=3, 2007, *Lophodytes cucullatus*); Common Merganser (n=17, 2007, *Mergus merganser*); and Ruddy Duck (n=1, 2008, *Oxyura jamaicensis*). AIV was not isolated from any of these species.

Table 3.2. Multivariable logistic regression model for the prediction of avian influenza virus isolation results in 4,563 waterfowl sampled in northwestern Minnesota, USA, 2007 and 2008.

Variable	Coefficient	†Robust SE	Odds Ratio (95% CI)	‡P
Year				< 0.001
2007 (1)	Referent			
2008 (2)	1.658	0.079	NC	
Location				< 0.001
Thief Lake WMA (1)	Referent			
Roseau River WMA (2)	-0.055	0.145	NC	
Agassiz NWR (3)	-2.270	0.101		
Week (centered)	1.163	0.043	NC	< 0.001
$(\text{Week})^2$	-0.392	0.006	NC	< 0.001
Age				< 0.001
Adult	Referent			
Juvenile	0.996	0.181	2.7 (1.4, 3.9)	
Species Type				< 0.001
Mallard	Referent			
Other Dabblers	-1.147	0.142	0.32 (0.24, 0.42)	
Divers & Wood Ducks	-2.313	0.407	0.10 (0.04, 0.22)	
Location X Week				< 0.001
2	-1.424	0.097	NC	
3	-0.362	0.101		
Location X $(\text{Week})^2$				<0.001
2	0.376	0.009	NC	
3	0.402	0.011		
Year X Location				< 0.001
2 2	-0.854	0.191	NC	
2 3	1.416	0.197		
Year X Week				< 0.001
2	-2.892	0.055	NC	
Year X Location X Week				< 0.001
2 2	3.078	0.102	NC	
2 3	1.810	0.126		
Constant	-2.674	0.160	---	< 0.001

†Robust standard error adjusted for clustering within capture sites (*n* = 24)

‡P-value based on Wald chi-square statistics

NC – Not calculated because the odds ratio depends on the level of the interacting variables

Table 3.3. Predominant hemagglutinin and neuraminidase subtype combinations detected in northwestern Minnesota, USA, 2007 and 2008, with a comparison to previous studies in North America and Europe.

Subtypes detected and percent (%)					
Alberta 1976-1990 (Sharp et al., 1997)	Alberta 1976-2001 (Krauss et al., 2004)	Northern Europe 1998-2007 (Munster et al., 2007)	2007 Minnesota	2008 Minnesota	Total Minnesota
H1N1 (2.6)	H1N1 (2.1)	H1N1 (6.0)	H1N1 (8.1)	H1N1 (1.6)	H1N1 (3.8)
H2N3 (0.7)	H2N3 (0.6)	H2N3 (4.2)	ND[†]	H2N3 (3.4)	H2N3 (2.3)
H3N8 (23.3)	H3N8 (22.8)	H3N8 (6.3)	H3N8 (15.3)	H3N8 (26.0)	H3N8 (22.4)
H4N6 (14.6)	H4N6 (12.5)	H4N6 (16.0)	H4N6 (20.7)	H4N6 (14.8)	H4N6 (16.8)
H5N2 (0.2)	H5N2 (0.2)	H5N2 (3.0)	H5N2 (0.5)	H5N2 (2.7)	H5N2 (2.0)
H6N2 (26.3)	H6N2 (20.8)	H6N2 (9.9)	H6N1 (3.2)	H6N1 (7.8)	H6N1 (6.2)
H7N3 (0.6)	H7N3 (0.7)	H7N7 (10.5)	H7N3 (5.4)	H7N3 (0.5)	H7N3 (2.1)
H8N4 (0.4)	H8N4 (0.3)	H8N4 (1.8)	H8N4 (0.5)	H8N4 (3.4)	H8N4 (2.4)
H9N1 (0.07)	H9N1 (0.1)	H9N2 (1.2)	ND	ND	ND
N10N7 (0.6)	N10N1, N10N6 (0.09)	N10N7 (1.2)	N10N7 (7.7)	N10N7 (5.5)	N10N7 (6.2)
H11N9 (0.6)	H11N9 (0.08)	H11N9 (4.8)	H11N9 (5.9)	H11N9 (0.2)	H11N9 (2.1)
N12N5 (0.3)	N12N5 (0.5)	N12N5 (1.2)	N12N5 (0.9)	ND	N12N5 (0.3)

[†] ND – Subtype not detected.

Table 3.4. Spatial variation in AIV subtypes from sites sampled in northwestern Minnesota, USA, 2007 and 2008.

	2007				2008			
Subtypes	Thief Lake WMA	Roseau River WMA	Agassiz NWR	n	Thief Lake WMA	Roseau River WMA	Agassiz NWR	n
H1N1	15	2		17	4		3	7
H2N2				0		1	1	2
H2N3				0	1	14		15
H3N1	6			6	6		4	10
H3N2				0	6	3	2	11
H3N6	6		4	10	11	9	9	29
H3N7				0			2	2
H3N8	1	16	12	29	20	28	66	114
H4N2				0	3	3	6	12
H4N4					1	1		2
H4N6	13	28	3	44	27	18	19	64
H4N8		1		1	14	5	11	30
H5N1				0	1	2		3
H5N2		1		1	3	2	7	12
H6N1		7		7	4	18	12	34
H6N2		4		4	2	7	6	15
H6N8		2		2	1	1	1	3
H7N3	10		1	11			2	2
H8N4		1		1		15		15
H10N6	3			3	1			1
H10N7	6	6	4	16	3		21	24
H11N9	9	2	2	13			1	1
H12N5	1	1		2				
Total	70	71	26	167	108	127	173	408

Table 3.S1. Summary of avian influenza virus isolation testing results for waterfowl sampled in northwestern Minnesota, USA, 2007 and 2008.

Variable	Sample Size	No. Positive (%)	†P
Year			
2007	2,441	222 (9.1)	< 0.001
2008	2,452	438 (17.9)	
Month			
July	471	82 (17.4)	< 0.001
August	832	118 (14.2)	
September	3,345	454 (13.6)	
October	245	6 (2.5)	
Species Type			
Mallards	3,117	531 (17.1)	< 0.001
Other Dabblers	1,221	120 (9.8)	
Divers & Wood Ducks	555	7 (1.3)	
Age			
Juvenile	3,170	543 (17.1)	< 0.001
Adult	1,723	117 (6.8)	
Location			
Thief Lake WMA	1,036	209 (20.2)	< 0.001
Roseau River WMA	1,820	216 (11.9)	
Agassiz NWR	1,707	232 (13.6)	
Fosston / Bemidji	330	3 (0.9)	
Total	4,893	660 (13.5)	

†Chi-square test of independence

Table 3.S2. Subtype combination found in all sampled duck species, all mallards, and juvenile mallards and their associated rate of recovery from the total number of birds sampled in northwestern Minnesota, USA, 2007 and 2008.

	2007						**2008**					
Subtypes	**All Species**	**Percent Prevalence**	**Mallards**	**Percent Prevalence**	**Juvenile Mallards**	**Percent Prevalence**	**All Species**	**Percent Prevalence**	**Mallards**	**Percent Prevalence**	**Juvenile Mallards**	**Percent Prevalence**
H1N1	18	0.74	17	1.00	11	1.26	7	0.29	4	0.28	3	0.28
H1N2	1	0.04	1	0.06	1	0.11						
H1N4	1	0.14	1	0.06								
H2N2							2	0.08	2	0.14	2	0.19
H2N3							15	0.61	15	1.05	13	1.22
H3N1	6	0.24	6	0.35	3	0.34	10	0.41	8	0.56	5	0.47
H3N2							11	0.45	8	0.56	8	0.75
H3N3	1	0.04	1	0.06	1	0.11	1	0.04	1	0.07	1	0.09
H3N4							1	0.04	1	0.07	1	0.09
H3N6	10	0.40	10	0.59	7	0.80	29	1.18	22	1.54	19	1.78
H3N7							2	0.08	2	0.14	2	0.19
H3N8	34	1.39	28	1.66	11	1.26	114	4.65	76	5.33	69	6.48
H3N9							1	0.04				
H4N2							12	0.49	9	0.63	8	0.75
H4N4							2	0.08	1	0.07	1	0.09
H4N5							1	0.04				
H4N6	46	1.88	24	1.42	17	1.95	65	2.65	53	3.72	45	4.23
H4N8	3	0.12	3	0.18	2	0.23	29	1.18	25	1.75	24	2.25
H5N1							3	0.12	2	0.14	1	0.09
H5N2	1	0.04	1	0.06	1	0.11	12	0.49	11	0.77	11	1.03
H6N1	7	0.29	7	0.41	7	0.80	34	1.39	28	1.96	26	2.44
H6N2	4	0.16	2	0.12	2	0.23	15	0.61	14	0.98	13	1.22
H6N4	1	0.04	1	0.06	1	0.11						
H6N6	1	0.04					1	0.04	1	0.07	1	0.09
H6N8	2	0.08	2	0.12	1	0.11	3	0.12	3	0.21	3	0.28
H7N3	12	0.49	12	0.71	7	0.80	2	0.08	2	0.14	1	0.09
H7N8	1	0.04	1	0.06								
H8N4	1	0.04	1	0.06			15	0.61	14	0.98	14	1.31
H10N1	1	0.04	1	0.06	1	0.11						
H10N2							1	0.04	1	0.07	1	0.09
H10N3	1	0.04	1	0.06	1	0.11						
H10N6	3	0.12	2	0.12	1	0.11	1	0.04				
H10N7	17	0.70	16	0.95	9	1.03	24	0.98	21	1.47	13	1.22
H10N8							1	0.04				
H11N1							1	0.04	1	0.07	1	0.09
H11N2	1	0.04	1	0.06	1	0.11	1	0.04	1	0.07	1	0.09
H11N6	1	0.04	1	0.06	1	0.11						
H11N9	13	0.53	12	0.71	11	1.26	1	0.04	1	0.07		
H12N5	2	0.08	2	0.12	1	0.11						
H12N9	1	0.04	1	0.06	1	0.11						

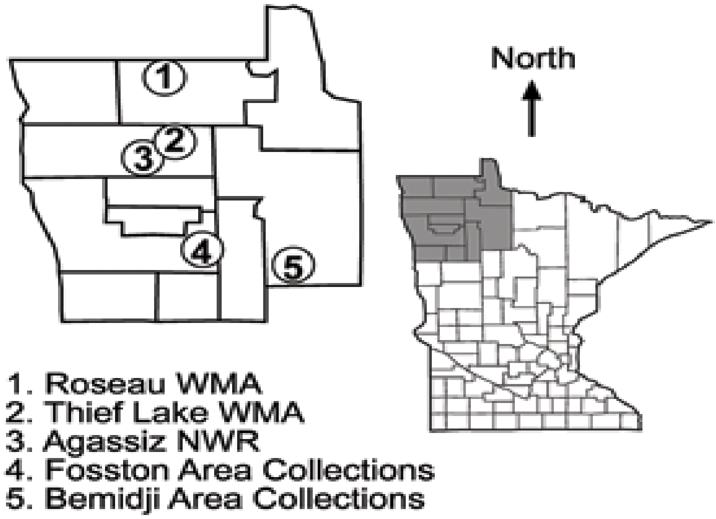

Figure 3.1. The location of sampled areas for avian influenza virus in northwestern Minnesota, USA, 2007 and 2008.

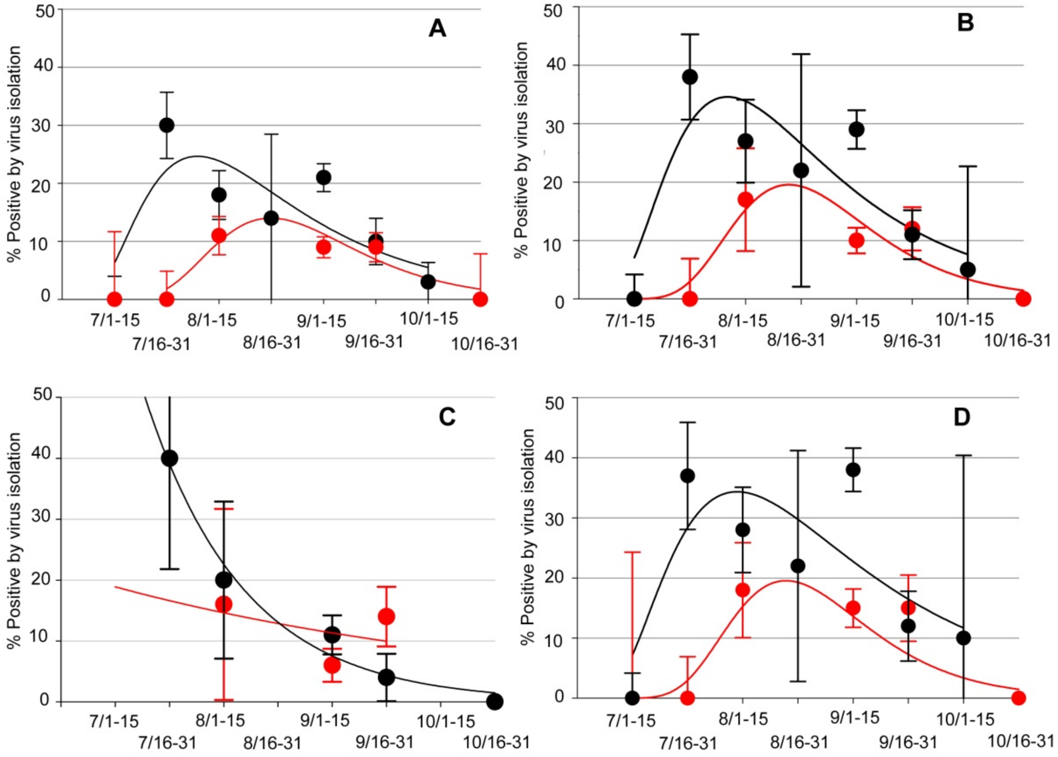

Figure 3.2: Epidemic curves showing the within-year temporal change in percent of ducks in northwestern Minnesota infected with avian influenza virus, 2007 (red lines) and 2008 (black lines; A = all species; B = Mallards; C = Adult Mallards; and D = Juvenile Mallards). Trend lines were calculated using SigmaPlot software (Systat Software Inc., Richmond California, USA) using a peak, log normal, 3 parameter function (A.B, and D) and exponential decay, single, 2 parameter functions (C).

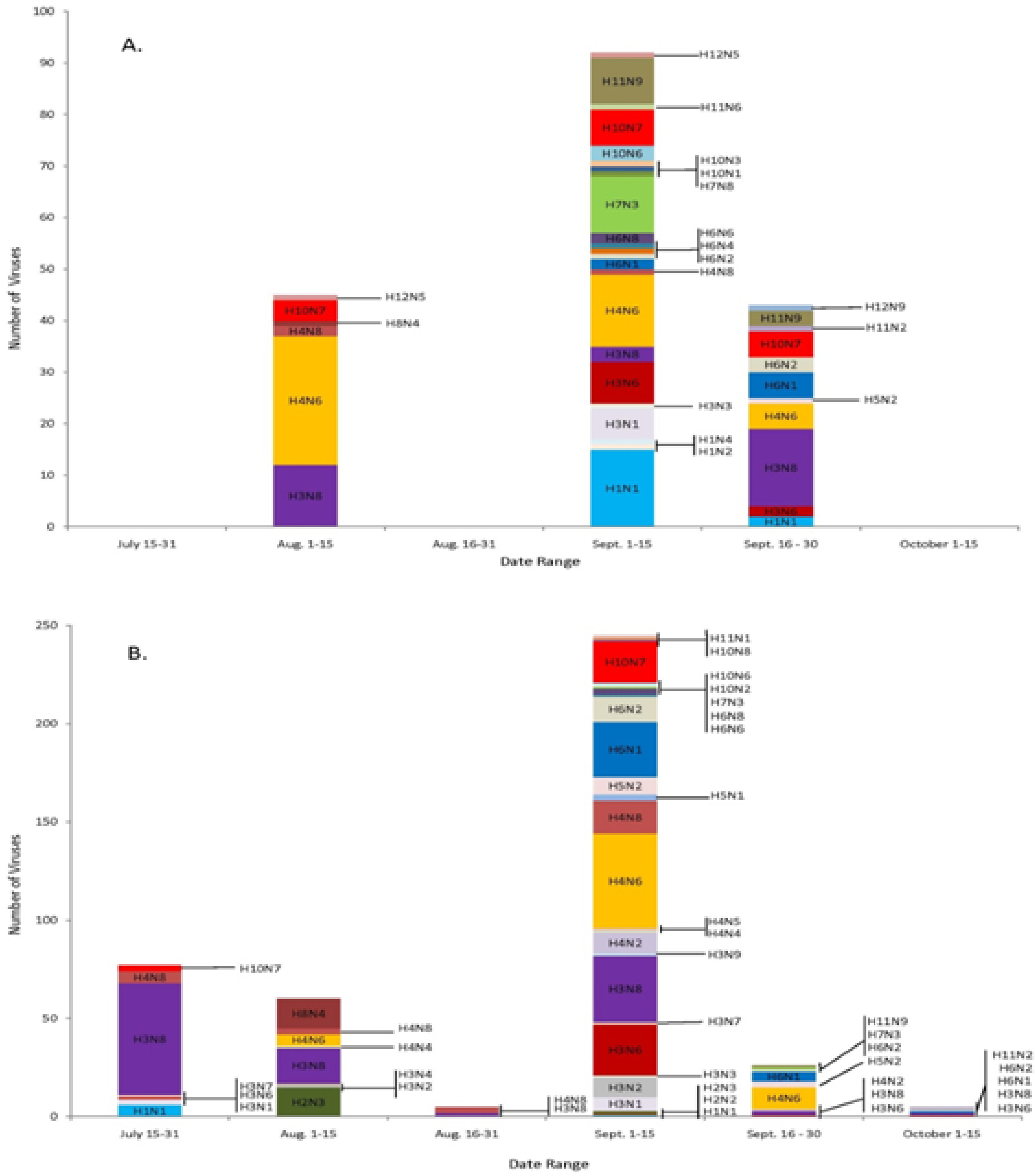

Figure 3.3: The temporal variation of subtype diversity of avian influenza viruses from ducks captured in northwestern Minnesota, USA (A = 2007; B = 2008). This graph demonstrates that the peak collection period to gather subtypes is early September.

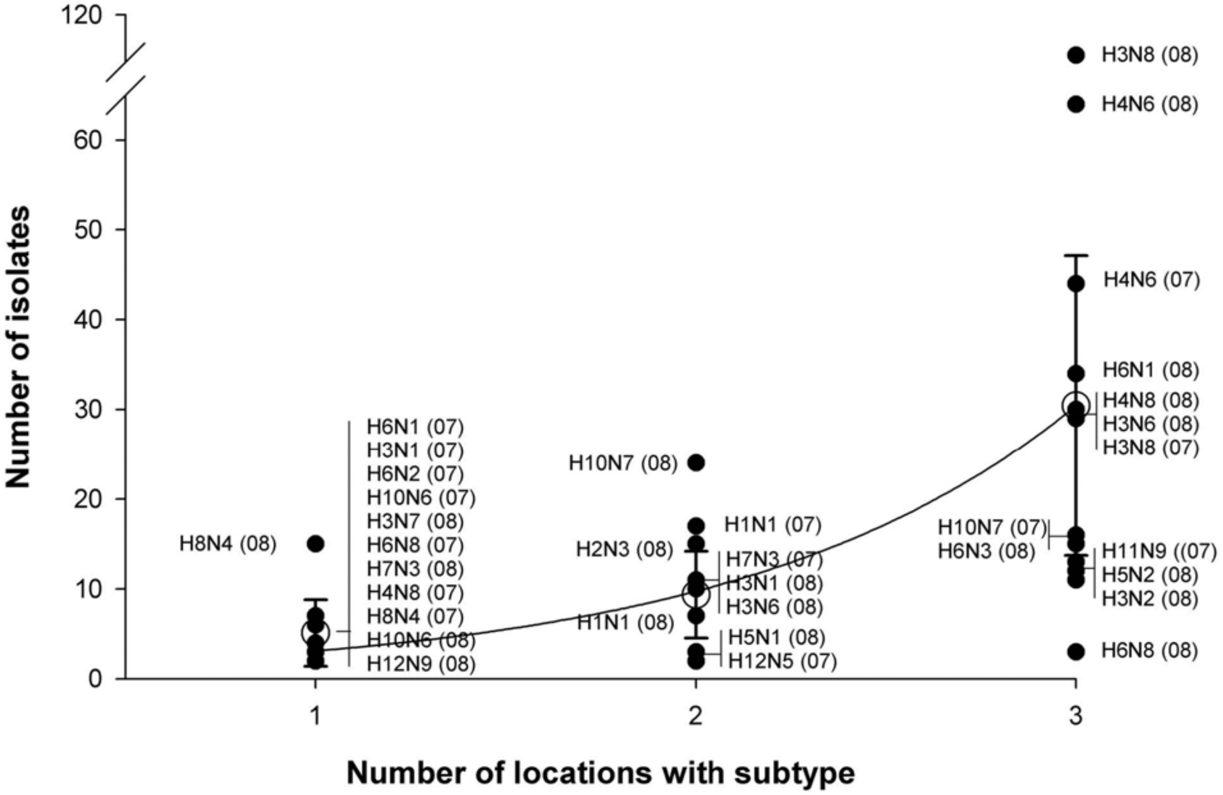

Figure 3.4: The number of subtype combinations of avian influenza virus that were detected in ducks captured at multiple sites in northwestern Minnesota, USA, 2007 and 2008.

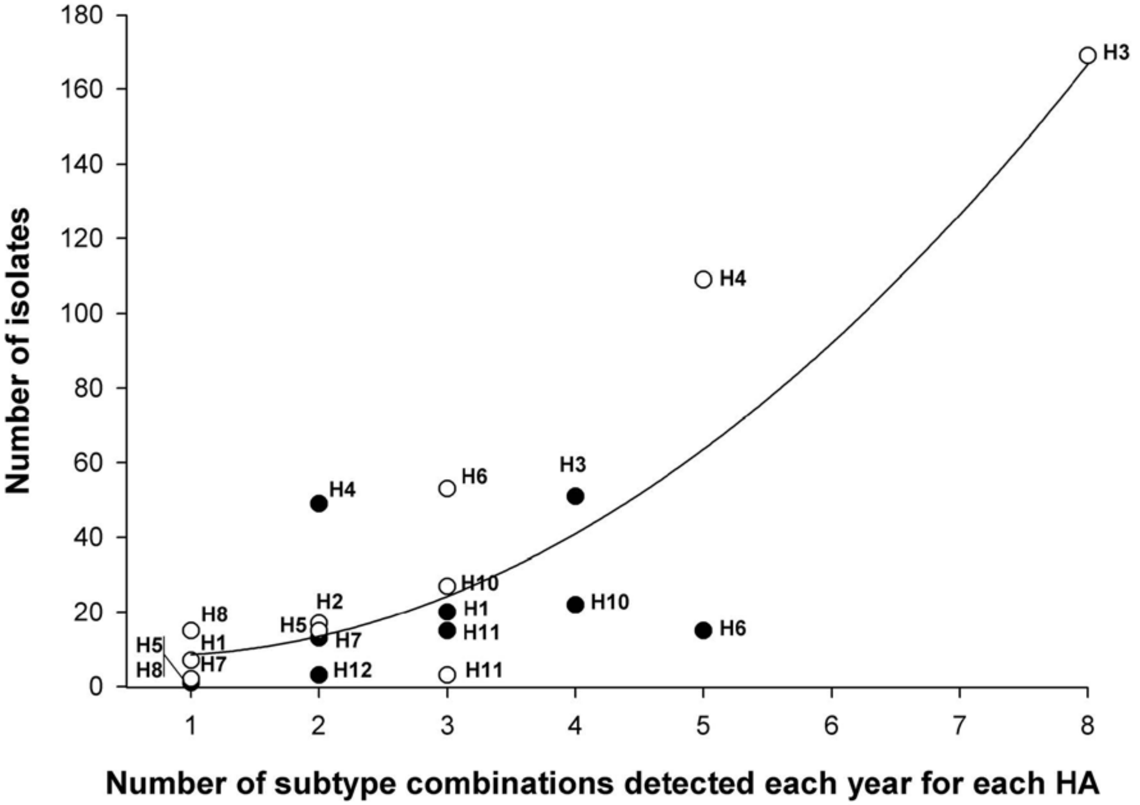

Figure 3.S1: The number of subtype combinations associated with each HA type and the number of those HA subtype detected during 2007 and 2008 (● = 2007 and ○ = 2008). The line is defined by the quadratic function *f*=9.4889-3.9055*x*+2.944 (*r^2=0.7607, P<0.0001).*

CHAPTER 4

CONCLUSIONS

Avian influenza virus (AIV) has become a disease that merits more research because of its threat to public health as seen by the outbreak of the highly pathogenic strain H5N1 in Asia. This study looked to further our understanding of AIV on a small spatial scale in northwestern Minnesota. Our goals were to: 1) describe within-season (mid-July to October) variation of AIV prevalence over a small geographic area; 2) determine the spatial, temporal, host, and interacting factors that influence prevalence in our study area; 3) determine the effects of location, time, and host on the detection of subtype diversity; and 4) evaluate sampling efforts necessary to obtain reliable surveillance data from a localized study.

Our research agreed with historic research both in North America and in Minnesota with regards to there being a late summer peak in AIV prevalence. The exact onset of that peak may vary slightly between years and between locations. We suggest that these differences may be observed because of migratory timing, population structure, or species abundance or diversity. We also suggest that more work be done to determine the effect that location has on transmission of AIV.

Mallards were the most likely species to be infected in our study which is also consistent with previous studies; however, numerous virus isolations were made from other species as well. We believe that although mallards are the most common ducks from which AIV are isolated, other species should not be discounted because they may play a role in virus transmission and maintenance. Also consistent with previous studies, we found that juvenile ducks were three time more likely to be infected with AIV than adults.

Though subtype diversity varied between years in our study, nearly all of the North American HA and NA subtypes were detected both years of our study with few exceptions. A peak in subtype diversity was detected in both years during the early part of September. We suggest that this increase in subtype diversity during this time may be because of increased detectability with decreased prevalence, migratory ducks moving into the area, or reassortment events.

We also suggest that you can adequately capture both prevalence and subtype diversity in northwestern Minnesota by only sampling juvenile mallards. This effort could greatly reduce the sample size. Since mallards were the predominate species caught, we are aware that the results were probably biased toward this species however the composition of the avian community that you are working in should be considered before undertaking any sampling. We also suggest that though subtype diversity could be captured by testing juvenile mallards, genetic diversity must also be looked at to be sure it is being captured as well.