INFLUENZA A VIRUSES AND AVIAN PARAMYXOVIRUSES IN WILD BIRDS: WHAT CAN SEROLOGY TEACH US?

by

WHITNEY MICHAEL KISTLER

(Under the Direction of Michael Yabsley)

ABSTRACT

Traditional surveillance of Influenza A viruses (IAVs) in wild birds has used RT-PCR or virus isolation. However, commercially available serological assays have been developed recently which can complement virus detection assays. This is useful because antibodies to IAVs are detectable longer than viral shedding and can be used in species that shed virus briefly or species that are not easily sampled when IAVs are circulating. In addition, serology has been an underutilized for avian paramyxovoviruses (APMVs) in wild birds. Furthermore, except for Newcastle disease virus (NDV), there is very few data on APMVs in wild birds. The objectives of this work were to use serological assays and virus isolation to determine: 1) IAV subtype-specific antibodies in Canada geese (*Branta canadensis*) and risk factors associated with exposure; 2) evaluate exposure of mute swans (*Cygnus olor*) to IAVs; and 3) the role of wood ducks (*Aix sponsa*) in the epidemiology of IAVs and APMVs.

Canada geese were frequently exposed to the same IAVs subtypes that circulate in dabbling ducks; however, they had a high prevalence of H5-specific antibodies which are rare in ducks. There was no significant variation in antibody prevalence among years and percent developed was the only environmental predictor variable associated with risk. These data suggest that Canada geese share a common exposure with dabbling ducks and serologic data are not

sensitive enough to detect local and annual variation in IAV circulation. Similarly, mute swans had a high IAVs antibody prevalence. The high nucleoprotein prevalence is likely related to antibody persistence and long life span of mute swans. The H5-specific antibody prevalence was also high which suggest mute swans may have flock immunity, which could protect them from disease associated with highly pathogenic H5N1.

Wood ducks were not frequently exposed to IAVs but are frequently exposed to APMVs. These data suggest exposure of wood ducks are likely only spillover hosts and exposure is location dependent and occurs only when ducks shed IAV. Additionally, wood ducks may be important hosts of APMVs, but virus isolation data is lacking.

INDEX WORDS: Avian Paramyxoviruses, Canada Geese, Influenza A Virus, Mute Swans, Serology, Wood Ducks

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CHAPTER 1

INTRODUCTION

The major objective of the studies included in this dissertation is to utilize serologic assays to better understand the epidemiology of influenza A viruses (IAVs) and avian paramyxoviruses (APMVs) in free-ranging waterfowl. There are numerous serotypes of IAVs and species of APMVs that are transmitted among waterfowl by the fecal-oral route (Webster et al., 1978; Deibel et al., 1983; Hinshaw et al., 1985; Olsen et al., 2006; Kim et al., 2012; Dai et al., 2013). Neither low pathogenic IAVs nor low pathogenic APMVs are associated with clinical disease in wild waterfowl; however, some serotypes of both viruses can cause clinical disease in poultry and other wild birds (Mustaffa-Babjee et al., 1974; Glaser et al., 1999; Alexander, 2000; Ellis et al., 2004; Hines and Miller, 2012). Studies on the ecology of these viruses are of great significant because of their importance to poultry health and their wide distribution.

Influenza A viruses have a large host range but birds in the orders Anseriformes and Charadiirformes serve as the reservoirs (Slemons et al., 1973; Bahl et al., 1975; Hinshaw et al., 1985; Krauss et al., 2004). Several IAV subtypes have spilled-over into new species which has resulted in pandemics and, in some cases, these viruses have become endemic in a novel host (Crawford et al., 2005; Smith et al., 2009; Anthony et al., 2012). Recent examples of pandemics include the emergence of the HPAI H5N1 viruses in chickens and people in Asia which spilled over into wild birds in Europe and Asia and the emergence of H1N1 in North America (Ellis et al., 2004; Neumann et al., 2009).

Traditionally, IAV surveillance in wild birds has been conducted using virus isolation in 9-11 day-old specific pathogen-free embryonated chicken eggs (Slemons et al., 1973;

Rosenberger et al., 1974; Webster et al., 1976). Although this is considered the most sensitive technique for detecting viable virus from wild birds, it requires birds to be actively shedding infectious virus, which can be brief and intermittent (Woolcock, 2008; Costa et al., 2010, 2011; Berhane et al., 2010). In addition, virus isolation is labor-intensive and expensive.

Historically, only two serological assays have been widely used to detect AIV antibodies in wild birds, the agar gel immunodiffusion (AGID) assay and hemagglutination inhibition (HI) assay (Laver et al., 1972; Slemons and Easterday, 1972; Winkler et al., 1972; Kocan et al., 1979). The AGID assay is commonly used in poultry; however, this assay lacks validation in many wild bird species and has a reported low sensitivity in waterfowl (Slemons and Easterday, 1972; Bahl et al., 1975; Brown et al., 2009). The HI assay is a subtype-specific serological assay thus serum samples must be tested against all 16 hemagglutinin (HA) subtypes to determine if a sample is negative (Assaad et al., 1980; Pedersen, 2008). Recently, a commercially available blocking enzyme-linked immunosorbent assay (bELISA) has been evaluated and shown to have high sensitivity and specificity for use in wild birds (Brown et al., 2009, 2010a, 2010b; Ishtiaq et al., 2012; Kistler et al., 2012). Because this bELISA targets antibodies directed to the nucleoprotein which is conserved across all 16 (HA) subtypes, this assay detects exposure to any IAV subtype (Walls et al., 1986).

Canada geese (*Branta canadensis*), a near ubiquitously distributed waterfowl species, have an undetermined role in the epidemiology of IAVs. Geese share aquatic habitats with waterfowl species considered to be reservoirs for IAVs and are susceptible to experimental infection with IAVs, but isolation of viruses from wild Canada geese is uncommon (Winkler et al., 1972; Hinshaw et al., 1986; Pasick et al., 2007; Berhane et al., 2010; Harris et al., 2010; Wilcox et al., 2011). However, a recent study showed that Canada geese are frequently exposed

to IAVs in the wild (Kistler et al., 2012). In addition to Canada geese, the role of wood ducks (*Aix sponsa*) and mute swans (*Cygnus olor*) in the epidemiology of IAVs is poorly understood. Wood ducks are experimentally susceptible to IAVs but field studies on wood ducks have been inconclusive. In a few studies, IAVs were isolated from wood ducks at certain locations and times but other large scale studies failed to detect viral shedding in wood ducks (Hinshaw et al., 1985; Slemons et al., 2003; Brown et al., 2007; Parmley et al., 2008; Goekjian et al., 2011). Two serological surveys did not detect IAV antibodies in wood ducks. One study sampled an unreported number of birds using the AGID assay and the other study used the more sensitive bELISA, but only tested three birds (Bahl et al., 1975; Brown et al., 2010a). Mute swans are an exotic and invasive species in North America. Only two studies have been conducted on mute swans in North America. Both of these studies isolated IAVs and detected antibodies to AIVs (Graves, 1992; Pedersen et al., 2014). Interestingly, IAVs usually associated with Charadriiformes has been isolated from mute swans and they have subtype specific antibodies to viruses that are not frequently detected in Anseriformes (Graves, 1992).

Avian paramyxoviruses are a diverse group of viruses which includes several viruses of importance to poultry (e.g., Newcastle disease virus (NDV), APMV-2, and APMV-3; Bradshaw and Jensen, 1978; Tumova et al., 1979). Only NDV is known to cause disease in wild birds in North America with numerous large epidemics occurring in double-crested cormorants (*Phalacrocorax auritus*) and die-offs in American white pelicans (*Pelicanus erythrorhynchos*) (Wobeser et al., 1993; Kuiken et al., 1998; Glaser et al., 1999). Wild waterfowl are considered reservoirs for low pathogenic NDV, APMV-4, APMV-6, and APMV-8 (Alexander, 1980; Alexander et al., 1983; Hinshaw et al., 1985; Stallknecht et al., 1991; Goekjian et al., 2011; Choi et al., 2013); however, because only NDV causes clinical disease in wild birds, few studies have

focused on APMV in waterfowl (Jindal et al., 2009). Only two studies in North America have used serologic testing to detect antibodies to APMVs in wild waterfowl and this study focused solely on NDV (Kocan et al., 1979).

Serologic testing can be a useful tool in understanding the role different species of birds play in the epidemiology of these viruses, but its use in waterfowl has been underutilized. Therefore, we propose to utilize serologic testing to increase our understanding of the role Canada geese, wood ducks, and mute swans (*Cygnus olor*) play in the epidemiology of IAVs and APMVs.

Specific objectives for of this study include:

1. To investigate if land use or other ecological factors predicts exposure of Canada geese (*Branta canadensis*) in Pennsylvania to IAVs
2. To determine if the AIV subtypes circulating in Canada geese are the same as those that circulate in sympatric waterfowl species using a subtype specific hemagglutination inhibition assay.
3. To investigate prevalence of H5-specific antibodies in free-ranging mute swans (*Cygnus olor*), using three serological assays.
4. Investigate the role of wood ducks (*Aix sponsa*) in the epidemiology of IAVs and APMVs using a combination of virus isolation and serologic testing

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CHAPTER 2

LITERATURE REVIEW

Influenza A Viruses

Influenza A viruses are in the family *Orthomyxoviridae*. Depending on the virus, the viral genome consists of eight segments of negative-sense ribonucleic acid, coding for 10-12 proteins (Compans et al., 1970; Rott, 1992; Suarez and Swayne, 2008; Jagger et al., 2012). Three of these proteins are found on the surface of the virion: hemagglutinin (HA), neuraminidase (NA), and the membrane ion channel (M2) (Compans et al., 1970; Krug and Etkind, 1973; Rott, 1992). The remaining proteins are expressed internally: nucleoprotein (NP), matrix protein (M1), polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), polymerase acidic protein (PA), nonstructural protein 1 (NS1), nonstructural protein 2 (NS2) (also called the nuclear export protein (NEP)), and in some IAV, the PB1-F2 protein, and a newly discovered protein PA-X (O'Neill et al., 1998; Chen et al., 2001; Suarez and Swayne, 2008; Jagger et al., 2012). The two nonstructural proteins are not usually found inside the virion, but are located within the nucleus (NS1) and cytoplasm (NS2) of the host cell (Krug and Etkind, 1973; Tumpey et al., 2005).

Influenza viruses are categorized by differences in three of these proteins. The NP protein is used to distinguish influenza types A, B, and C from each other and shows little antigenic variation within a particular influenza type (Walls et al., 1986; Webster et al., 1992; Olsen et al., 2006). The M1 protein is also conserved among all IAVs (Schild, 1972). Because the NP and M1 proteins are relatively conserved among influenza A viruses they are used often in molecular and serological assays to detect these viruses in a wide range of bird and mammal species (Schild,

1972; Yewdell et al., 1985; Fouchier et al., 2000; Spackman et al., 2002; Brown et al., 2009). However, classification of these viruses to subtypes (e.g., H3N8) is important for epidemiologic studies; therefore, researchers exploit variations in the HA and NA proteins (Assaad et al., 1980; Webster et al., 1992; Olsen et al., 2006).

Classification of Influenza A Viruses in Birds

Currently, 16 H subtypes (H1-16) and 9 N subtypes (N1-9) have been identified in influenza viruses circulating among wild birds (Webster et al., 1992; Olsen et al., 2006). In poultry, IAVs are further classified based on the severity of disease which determine if viruses are categorized as high pathogenic or low pathogenic (HPAIV and LPAIV, respectively). To date, only H5 and H7 viruses have developed into HPAIV and these HPAIV are believed to have arisen due to mutations of LPAIV that occurred during transmission among domestic birds (Horimoto et al., 1995; Alexander, 2000). For a virus to be classified as HPAIV two tests can be used including a chicken challenge test and sequence analysis of the HA cleavage site (OIE, 2012). The chicken challenge test can be done either by challenging 6-week-old chickens with the virus then calculating the intravenous pathogenicity index (IVPI), which is the the severity of disease caused by the virus inoculated intravenously, or by measuring the mortality in 4- to 8-week old chickens. For the virus to be considered highly pathogenic, the IVPI index must be >1.2 or the mortality rate must be $\geq$75% in inoculated chickens. In addition to these live chicken trials, a virus can be classified as highly pathogenic if there is an insertion of 2 or more basic amino acids (lysine and arginine) in the HA cleavage site sequence. The OIE only requires one of these assays to classify an IAV as a high pathogenic virus (OIE, 2012) and contradictory results between the tests have been reported only in Texas in 2004 (Lee et al., 2005).

Importantly, the classification of a virus as HPAI or LPAI is based on its effects in poultry and does not reflect how the virus will act in other species. For example, some highly pathogenic poultry viruses showed no signs of disease in experimentally inoculated ducks (Alexander et al., 1986).

Host Range of Influenza A Viruses

Influenza A virus have been reported from >100 bird species from 13 avian orders; however, most isolates have been acquired from aquatic birds in the orders Anseriformes and Charadriiformes (Stallknecht and Shane, 1988; Olsen et al., 2006). Although wild birds are considered the natural reservoirs, several subtypes have crossed the species barrier and have become established in new hosts. Humans (*Homo sapiens*), dogs (*Canis lupus familiaris*), horses (*Equus ferus*), and swine (*Sus scrofa*) are all examples of species that have IAVs that have adapted and become endemic in their populations (Crawford et al., 2005; Kayali et al., 2008; Schnitzler and Schnitzler, 2009; Nelson et al., 2012). In addition to crossing species barriers and becoming endemic in new hosts, IAVs can spillover into new hosts resulting in pandemics or small outbreaks in new hosts. During that last two decades, two avian IAVs (H5N1 and H7N9) have infected humans causing severe disease with high case fatality rates (Claas et al., 1998; Lebarbenchon et al., 2013a; To et al., 2013) and in 2009, a triple assortment swine IAV (H1N1) crossed into humans causing a pandemic that subsequently became endemic in the human population (Smith et al., 2009; Wong et al., 2012). Importantly, these pandemics are not restricted to humans. In 2011, a H3N8 virus caused severe pneumonia and die-offs in New England harbor seals (*Phoca vitulina*). Although not associated with mortality, two novel IAVs (subtypes H17N10 and H18N11) have been detected in bat species (Tong et al., 2012, 2013).

Although wild birds from the orders Anseriformes and Charadriiformes are recognized as the major reservoirs for all subtypes of AI viruses, there is considerable variation in prevalence among the species within these orders (Deibel et al., 1983; Hinshaw et al., 1985; Kawaoka et al., 1990; Krauss et al., 2004; Hanson et al., 2005, 2008; Velarde et al., 2010; Hoye et al., 2010; Wilcox et al., 2011) For example, within the order Anseriformes, most virus isolates come from species in the family Anatidae, genus *Anas*, particularly mallards (*Anas platyrhynchos*) (Stallknecht and Shane, 1988; Olsen et al., 2006; Stallknecht and Brown, 2007) and among the Charadriiformes, most virus isolations have been from members of the family Scolopacidae, particularly from ruddy turnstones (*Arenaria interprets*) (Sharp et al., 1993; Krauss et al., 2004; Hanson et al., 2008; Bahl et al., 2013). However, in the family Laridae, high virus isolation rates (>20%) have been detected in nesting colonies of ring-billed gulls (*Larus delawarensis*) and black-headed gulls (*Chroicocephalus ridibundus*) (Velarde et al., 2010; Verhagen et al., 2014).

Similarly, the subtypes of AIV detected in birds in Anseriformes and Charadriiformes are not equally distributed among the species. In the family Anatidae, particularly in the genus *Anas*, the predominant AIV subtypes isolated include H3, H4, and H6, with sporadic detections of most other subtypes (Deibel et al., 1983; Hinshaw et al., 1985; Sharp et al., 1997; Krauss et al., 2004; Ip et al., 2007; Munster et al., 2007; Kulak et al., 2010). However, there is some variation in the predominant subtypes as certain ones have been detected in higher frequency at some locations and in some years (Stallknecht et al., 1990b; Slemons et al., 1991; Hanson et al., 2005; Baumer et al., 2010; Goekjian et al., 2011; Wilcox et al., 2011; Ferro et al., 2012; Tolf et al., 2012; Vittecoq et al., 2012). Interestingly, H16 has not been isolated from Anatidae and H13 has rarely been isolated from these birds (Fouchier et al., 2005; Munster et al., 2007). In contrast, the subtypes most frequently isolated from Charadriiformes include the H3, H11, H13, and H16

subtypes (Graves, 1992; Krauss et al., 2004; Munster et al., 2007; Hanson et al., 2008; VanDalen et al., 2008; Velarde et al., 2010).

Determinants for Influenza A Virus Host Range

Factors that determine the host range for IAV are not fully understood; however, the hemagglutinin (HA) protein has a large role in determining host range. The HA protein is responsible for the virus binding to receptors on the host cell (Nelson et al., 1993; Skehel and Wiley, 2000). The HA binds to sialyloligosaccharide of the host cell and viruses adapted to birds preferentially bind to N-acetylneuraminic acid-α2,3-galactose while human viruses preferentially bind to N-acetylneuraminic acid-α2,6-galactose (Rogers and Paulson, 1983). These binding affinities also results in differential tissue tropism because, in the gastrointestinal tract of birds, cells primarily express N-acetylneuraminic acid-α2,3-galactose whereas cells in the human respiratory tract primarily express N-acetylneuraminic acid-α2,6-galactose (Ito and Kawaoka, 2000; Shinya et al., 2006; Jourdain et al., 2011; França et al., 2013). However, humans do have N-acetylneuraminic acid-α2,3-galactose receptors on cells lining the lower respiratory tract; therefore they can be directly infected with avian viruses as seen with H5N1 (Claas et al., 1998; Shinya et al., 2006). Additionally, swine and some avian species have respiratory cells with both receptors which may allow them act as mixing vessels of human and avian-adapted viruses (Ito et al., 1998; Thontiravong et al., 2012).

Influenza A viruses have high mutation rates because they are RNA viruses (Drake, 1993; Nobusawa and Sato, 2006). The ability of IAVs to mutate and change hosts has been frequently documented and this random mutation is called antigenic drift (Gerhard and Webster, 1978; Both et al., 1983). In addition to antigenic drift, IAVs can undergo antigenic shift, which is

the recombination of any of their eight genome segments when an individual cell is infected with two or more strains of virus (Dong et al., 2011; Kang et al., 2012; Zhao et al., 2012; Lebarbenchon et al., 2012b; Lu et al., 2014). Antigenic shift can result in IAVs quickly adapting to new hosts. Often, these host shifts can lead to pandemics as seen with H7N9 viruses in Asia and H1N1 in North America (Neumann et al., 2009; Lebarbenchon et al., 2013a; Lam et al., 2013).

Transmission of Influenza A Viruses in Wild Birds

Historically, based on data from domestic birds, IAVs in wild birds were thought to be primarily respiratory pathogens; however, during surveillance of wild birds, more isolations were obtained from cloacal samples compared with trachea samples (Slemons et al., 1973; Rosenberger et al., 1974; Bahl et al., 1975; Webster et al., 1976). Subsequent experimental infection studies showed that these viruses replicated within the digestive tract of ducks (Slemons and Easterday, 1977; Webster et al., 1978). In addition, Webster et al. (1978) showed that IAVs were shed in feces and they remained infective in feces for at least two weeks. Other field studies also found that viruses could be isolated from feces and water (Hinshaw et al., 1979; Ito et al., 1995). Hence, in a natural environment transmission of IAVs is likely via the fecal-oral route through ingestion of water and soil contaminated with feces. Experimentally, transmission has been proven through direct contact of infected and non-infected birds, through aerosols, and by contact contaminated water (Homme and Easterday, 1970; Winkler et al., 1972; Slemons and Easterday, 1977; Markwell and Shortridge, 1982; Forrest et al., 2010). Additionally, IAVs have been isolated directly from feathers and swabs of feathers, suggesting that preening is a possible IAV transmission route (Delogu et al., 2010; Lebarbenchon et al., 2013c). Transmission by direct

contact and aerosols probably do not play an important role in Anseriformes; however, they may play a more important role in high density nesting colonies of Charadriiformes where virus have been detected in >20% of birds (Velarde et al., 2010; Verhagen et al., 2014).

Spatial and Temporal Variation in Wild Birds

In addition to differences in viral subtype associations among the Anseriformes and Charadriiformes, location and seasonality of viral transmission is unique among the two orders. Within the Anseriformes (primarily mallard ducks), prevalence of viral shedding peaks in the northern United States and Canada when waterfowl are in high density staging flocks preparing for fall migration (Hinshaw et al., 1978, 1985; Deibel et al., 1983; Sharp et al., 1993; Krauss et al., 2004; Wilcox et al., 2011). However, prevalence decreases sharply in October and November as birds migrate south and prevalence remains low while birds are on the wintering grounds (Kocan et al., 1980; Smitka and Maassab, 1981; Stallknecht et al., 1990a; Ferro et al., 2012). The late summer and early fall spike in prevalence is likely due to the influx of large numbers of susceptible hatch-year birds at these pre-migration staging areas which have significantly higher virus isolation rates during fall sampling (Sharp et al., 1993; Alfonso et al., 1995; Hanson et al., 2003; Wilcox et al., 2011). Interestingly, this peak in viral shedding is observed annually in northern areas of North America every year during late summer and early fall, but there are some subtypes that are transmitted on a two to four year cycle in Alberta, Canada (Sharp et al., 1993; Krauss et al., 2004). Finally, exceptions to this typical transmission has been observed in which increased viral shedding rates have been reported in dabbling ducks in late winter in Texas and in the spring in Alaska (Hanson et al., 2005; Ip et al., 2007).

AIV transmission among the Charadriiformes is markedly different. There is only a single location in North America (Delaware Bay, USA), where IAV are consistently isolated in high prevalences (>5%) from Charadriiformes (primarily ruddy turnstones) and these prevalence rates tend to be higher in birds using Delaware Bay as a stopover during the spring migration (Kawoaka et al., 1988; Krauss et al., 2004; Hanson et al., 2008). In Charadriiformes outside of Delaware Bay, viral shedding is usually detected in low amounts (Ip et al., 2007; Munster et al., 2007; Hanson et al., 2008; Verhagen et al., 2012; Van Borm et al., 2012); however, in nesting colonies of ring-billed gulls and black-headed gulls virus isolation prevalence has been >20% (Velarde et al., 2010; Verhagen et al., 2014).

Influenza A Virus Serology

Historically, two serologic assays, the agar gel immunodiffusion (AGID) and hemagglutination inhibition (HI) assays, have been the most widely used assays for the detection of AIV antibodies in wild birds (Winkler et al., 1972; Bahl et al., 1975, 1977; Nettles et al., 1985; Brown et al., 2009). The AGID assay, considered the gold standard in poultry, has not been validated for use in most wild bird species and has poor reported sensitivity in waterfowl species (Beard, 1970; Slemons and Easterday, 1972; Bahl et al., 1975; Brown et al., 2009). The HI assay requires screening against all 16 hemagglutinin subtypes before presence or absence of antibodies can be determined, making large scale surveillance studies impractical. Recently, several enzyme-linked immunosorbent assays (ELISA) have been developed for the detection of AIV antibodies in a wide range of avian species (Jin et al., 2004; Pasick et al., 2007; Brown et al., 2009, 2010a; Sullivan et al., 2009; Lebarbenchon et al., 2012a; Claes et al., 2012).

These ELISAs have increased sensitivity for IAV antibody detection compared to the AGID assay (Brown et al., 2009; Sullivan et al., 2009). However, many of these ELISAs are not commercially available (Jin et al., 2004; Pasick et al., 2007) making quality control and comparability among different laboratories difficult. Four commercially available ELISAs have been used to test wild birds for AIV antibodies; however, three of these assays (Ingezim Influenza A(R), (Ingenasa, Spain), influenza A antibody competition(R) (IdVet, France), and Flu Detect BE Avian Influenza Antibody Test(R) (Synbiotics, Kansas City, Missouri)) have not been thoroughly validated for use with wild bird species (Pérez-Ramírez et al., 2010; Claes et al., 2012). Although all three have been used in surveillance of wild birds, they have only been used to analyze <100 samples of naturally exposed birds without testing experimentally infected birds. The fourth ELISA, the Flockcheck AI multiS-screen antibody test kit® (IDEXX, USA) is very sensitive 100% (95% CI: 96.5, 100.0) and specific 86% (95% CI: 75.6, 87.4) in detecting antibodies in numerous experimentally infected wild bird species from 10 taxonomic orders, including the Anseriformes and Charadriiformes (Brown et al., 2009). Furthermore, this IDEXX assay has been used in several large scale wild bird AIV surveillance and has outperformed the AGID assay in both experimentally- and naturally-exposed birds (Brown et al., 2010b; Lebarbenchon et al., 2012a; Ishtiaq et al., 2012; Kistler et al., 2012). Although, the IDEXX ELISA is effective at detecting antibodies in wild birds, it does not provide information on subtype specific antibodies.

The HI assay is still the most commonly used assay to detect subtype specific antibodies in wild birds (Hlinak et al., 2009; Berhane et al., 2010; Kruckenberg et al., 2011; Sousa et al., 2013; Berhane et al., 2014). However, the HI assay relies antibodies to bind to IAV antigen to inhibit viral binding to red blood cells (usually chicken red blood cells) so sera from some

species can cause non-specific inhibition of red blood cells, leading to false positive results (Hilleman and Werner, 1953; Hartley et al., 1992). These inhibitors can be removed by treating sera with either a receptor destroying enzyme, heat inactivation, adsorption with red blood cells, perodate, or Kaolin (Springer and Ansell, 1958; Ryan-Poirier and Kawaoka, 1991; Subbarao et al., 1992; Kim et al., 2012a). These serum inhibitors are most important when testing samples from mammals (Hilleman and Werner, 1953; Ryan-Poirier and Kawaoka, 1991; Hartley et al., 1992). In addition to the HI assay, several subtype specific ELISAs have been recently developed; however, only one (ID Screen Influenza H5 Antibody Competition(R) (IDVET, France)) has been evaluated in experimentally infected waterfowl (Lebarbenchon et al., 2013b). This IDVET H5 ELISA performed well in experimentally infected waterfowl; however, the protocol had to be modified from the manufacturers protocol to perform adequately.

Historically, IAV serological studies were largely exploratory using HI assays to better define subtypes wild birds were exposed and antibody response after experimental infections (Easterday et al., 1968; Slemons and Easterday, 1972; Winkler et al., 1972; Hinshaw et al., 1985). Although not used frequently in wild birds, some studies used the AGID assay to detect precipitating antibodies to the matrix protein of IAVs (Bahl et al., 1975; Kocan et al., 1979; Stanislawek et al., 2002; Harris et al., 2010). This assay was used to better understand, which wild bird species were involved in the epidemiology of IAVs; although, the AGID assay was considered the gold standard in poultry, it performed poorly in wild waterfowl species (Beard, 1970; Brown et al., 2010a, 2010b). However, some studies still used the AGID to determine exposure of rare or endangered species (Nolting et al., 2013). As the interest in IAVs in wild birds grew there was a push to develop better serological assays to detect antibodies to IAVs across multiple species. This led to several commercial and academic laboratories to develop

blocking (competitive) ELISAs to detect IAV antibodies across multiple species (Jin et al., 2004; Pasick et al., 2007; Brown et al., 2009; Curran et al., 2013). These ELISAs led research focusing on validating these assays with sera from experimentally infected birds and wild birds with comparison to other assays (e.g. AGID) (Pasick et al., 2007; Pérez-Ramírez et al., 2010; Brown et al., 2010a; Claes et al., 2012; Lebarbenchon et al., 2013b). Once ELISAs were validated, studies began to focus on sampling wild birds that have not been traditionally associated with IAVs and on geographic areas where virus isolations cannot easily be done (Brown et al., 2010b; Ishtiaq et al., 2012; Lewis et al., 2013). Serological studies also began to focus on species that had been frequently sampled for virus isolation. This was done to better define the roles of species with few virus isolations played in the epidemiology of IAVs and to set a baseline to compare known reservoirs species to species whose exposure to IAVs is unknown (Brown et al., 2010a; Kistler et al., 2012). Experimental infection studies have used serological assays to better understand host response to infections. This has been done with single exposure and looking at the onset and duration of detectable antibodies (Pasick et al., 2007; Ferreira et al., 2010; Costa et al., 2011). Currently, field and experimental studies are using serology to understand how multiple infections and heterosubtypic immunity affect host immune response and drive IAV infection dynamics (Berhane et al., 2010, 2014; Fereidouni et al., 2010; Costa et al., 2010; Latorre-Margalef et al., 2013; Tolf et al., 2013).

Influenza A Viruses in Canada Geese

Numerous previous surveillance studies have tested Canada geese (*Branta canadensis*) for IAVs (Tables 1 and 2). These studies indicate that Canada geese are naturally- and experimentally-susceptible to infection with IAVs (Rosenberger et al., 1974; Nettles et al., 1985;

Pasick et al., 2007; Berhane et al., 2010, 2014) but virus isolation rates are lower when compared to other members of the Anatidae family (i.e. genus *Anas*) collected at the same time and locations (Hinshaw et al., 1985; Ip et al., 2007). Most importantly, these studies indicate that naturally-exposed geese develop a detectable antibody response (Easterday et al., 1968; Harris et al., 2010; Kistler et al., 2012). Experimental inoculations of Canada geese with IAVs showed similar results. Experimentally inoculated birds seroconverted, but shed low levels of detectable virus for only a short duration (Homme and Easterday, 1970; Winkler et al., 1972; Pasick et al., 2007; Berhane et al., 2010, 2014). Experimentally, infected Canada geese have higher shedding prevalences and duration after infection with H5 subtype viruses than after inoculation with other subtypes; however, duration of viral shedding is <6-days (Berhane et al., 2010, 2014). In contrast, experimentally infected dabbling ducks shed virus for 10 days or longer (Costa et al., 2011; Brown et al., 2012). Collectively, these results indicate that Canada geese are susceptible to infection with LPAI viruses, survive the infection, and seroconvert, but have only minimal viral shedding. Thus they are not likely important reservoirs or amplifying hosts for IAV.

Recent experimental infection studies of IAVs in Canada geese have provided some very valuable information on IAVs in geese. These studies indicate that infection with low pathogenic viruses usually requires multiple infections to provide a subtypes specific immune response and that some subtypes (i.e., H5) replicate better and elicit a more robust subtype-specific immune response that other subtypes (Berhane et al., 2010, 2014). These studies also showed that previous exposure to low pathogenic IAVs can be protective in reducing morbidity from infection with highly pathogenic H5N1 (Pasick et al., 2007; Berhane et al., 2010, 2014). They also demonstrated that antibodies to low pathogenic H5 hemagglutinin and N1 neuraminidase

proteins could likely provide some level of protective immunity to infection with high pathogenic H5N1 (Berhane et al., 2010, 2014).

Table 2.1. Studies that have tested Canada geese for antibodies to IAVs

Reference	Assay	Positive/Sampled (%)
Easterday et al. 1968	HI[a]	8/12 (66)
Winkler et al., 1972	HI	66/1,401 (5)
	AGP[b]	8/1,359 (0.6)
Bahl et al. 1977	HI and AGP	0/65 (0)
Nettles et al. 1985	HI and AGP	90/261 (34)
Graves, 1992	HI and EI[c]	4/28 (14)
Pasick et al., 2007	ELISA	10/24 (42)
Harris et al., 2010	AGP	4/336 (1.2)
Claes et al., 2012	ELISA	36/73 (49)
Kistler et al., 2012	ELISA	483/3,205 (15)

[a]Hemagglutination inhibition assay
[b]Agar gel precipitin assay (Agar gel immunodiffusion assay)
[c]Elution inhibion assay

Table 2.2. Virus isolation results from Canada geese.

Reference	Positive/Sampled (%)
Rosenberger et al. 1974	1/52 (2)
Bahl et al., 1977	0/65 (0)
Boudreault et al., 1980	7/4 (57)
Smitka et al. 1981	0/11 (0)
Deibel et al. 1983	0/275 (0)
Nettles et al. 1985	2/1,504 (0.3)
Hinshaw et al. 1986	0/277 (0)
(Slemons et al., 1991)	0/315 (0)
Graves, 1992	0/348 (0)
Alfonso et al. 1995	0/5 (0)
(Ito et al., 1995)	4/663 (0.6)
Ip et al. 2008	4/249 (2)
(Pannwitz et al., 2009)	1/97 (1)
(Harris et al., 2010)	0/1,668 (0)
Total	22/6,026 (0.4)

Influenza A Viruses in Wood Ducks

Wood ducks (*Aix sponsa*) have been sampled for IAVs in several studies; however, their role in the epidemiology of IAVs is still unclear. Most of these studies sampled <60 individuals and did not isolate any IAVs (Rosenberger et al., 1974; Bahl et al., 1975; Webster et al., 1976; Kocan et al., 1979; Nettles et al., 1985; Hinshaw et al., 1986; Slemons et al., 1991). Three studies included sample sizes >100, Deibel et al. (1983) isolated AIVs from 16 of 748 wood ducks, Wilcox et al., (2011) isolated a single IAV from 206 wood ducks, and Goekjian et al. (2011) did not isolate any IAVs from 348 ducks. Two more studies detected increased (>8%) viral shedding in wood ducks (Hinshaw et al., 1985; Slemons et al., 2003). Slemons et al., (2003) detected viral shedding in 11% of wood ducks during a 1-week window and Hinshaw et al., (1985) isolated IAVs from 8% of wood ducks. One study conducted in Canada detected virus in 68% (71/104) wood ducks. This study used RT-PCR, which is more sensitive than virus isolation but does not ensure viable virus was detected (Parmley et al., 2008). Only two studies have attempted to detect IAV antibodies in wild wood ducks and although both failed to detect any antibodies, one study only sampled three birds (Brown et al., 2010a) and the other study used the less sensitive AGID assay (Kocan et al., 1979).

Experimental infection studies with wood ducks have been conducted using both high pathogenic IAVs and low pathogenic IAVs. Wood ducks were susceptible to high pathogenic IAVs, shed detectable amounts of virus, and developed antibodies as a result of infection (Brown et al., 2007, 2009). Wood ducks also showed a high level of morbidity and mortality after infection with HP H5N1. After inoculation with low pathogenic IAVs, wood ducks shed virus mainly via the oropharyngeal route and developed detectable antibodies (Costa et al., 2011). Collectively, these data indicated that wood ducks are susceptible to infection and seroconvert;

however, viral shedding in naturally occurring populations appears to be very restricted both temporally and spatially.

Influenza A Viruses in Mute Swans

In North America, only two studies have tested free-ranging mute swans (*Cygnus olor*) for IAVs and both detected a low prevalence (<1%) of virus shedding (Graves, 1992; Pedersen et al., 2014). Both studies also tested for antibodies to IAVs. Graves (1992) detected antibodies to H2, H5, H6, and H11 subtypes in >30% of mute swans in Maryland using the HI assay. Interestingly, the single virus isolate obtained by Graves (1992) was an H13N6 subtype and swans had antibodies to this subtype. This same subtype was also detected in ring-billed gulls during the same year and location (Graves 1992). Pedersen et al., (2014) detected isolation of IAVs from 31 of 390 (0.8%) mute swans from Massachusetts, Michigan, New Jersey, New York, Rhode Island, and Wisconsin. Antibodies to AIV were detected in 45% of 344 mute swans, but because birds were tested with a commercial bELISA (IDEXX), no subtype data was available.

Mute swans are native to Europe and are a large conspicuous bird that were frequently found dead during outbreaks of highly pathogenic H5N1 in Europe and Asia (Nagy et al., 2007; Starick et al., 2008; Globig et al., 2009; Feare, 2010; Fink et al., 2010; Pybus et al., 2012). These studies show that mute swans are susceptible with highly pathogenic H5N1 infections and morbidity and mortality are often seen during these outbreaks (Teifke et al., 2007; Globig et al., 2009; Nagy et al., 2012). The morbidity and mortality associated with highly pathogenic H5N1 in wild mute swans has also been reported in in experimentally infected birds (Brown et al., 2008; Kalthoff et al., 2008). A highly pathogenic H5N1 outbreak was documented in a heavily

monitored flock of mute swans in the United Kingdom and interestingly most of the birds that died were young birds (<3-years-old) (Pybus et al., 2012). Because these birds were heavily monitored, serum samples were available from previous years, and testing indicated that older birds were more likely to have antibodies which suggested that previous exposure to IAVs may have provided protection to infection with highly pathogenic H5N1.

Avian Paramyxoviruses

Avian paramyxoviruses (APMVs) are single stranded, negative sense RNA viruses from the genus *Avulavirus* (Lamb and Parks, 2013). To date, 13 avian paramyxoviruses have been identified from wild birds and they are classified as Newcastle Disease Virus (NDV; pseudonym APMV-1) and APMV-2 - APMV-12) (Miller et al., 2010; Dundon et al., 2012; Lamb and Parks, 2013). These viruses contain six genes that code for six proteins: nucleoprotein, phosphoprotein, matrix protein, fusion protein, large polymerase protein and hemagglutinin-neuraminidase protein (Lamb and Parks, 2007) (Subbiah et al., 2008; Samuel et al., 2009, 2010; Lamb and Parks, 2013), with the exception of APMV-6, which contains an additional small hydrophobic gene encoding for a small hydrophobic protein (Chang et al., 2001).

Avian paramyxoviruses are classified using hemagglutination inhibition assay (Alexander, 1980; Alexander et al., 1983; Alexander and Senne, 2008). Because Newcastle Disease Virus is an important pathogen of domestic poultry, considerable work has been done on this virus (Alexander et al., 2012). Like certain subtypes of IAVs, serotypes of APMVs are considered highly pathogenic. The other APMVs are less well studies; however, APMV-2 and APMV-3 can cause disease in poultry (Bradshaw and Jensen, 1978; Tumova et al., 1979). Avian paramyxovirus-5 is unique among APMVs because unlike the other APMVs it cannot be

cultured in embryonated chicken eggs and has only been reported from outbreaks of disease in Budgerigars (*Melopsittacua undulates*) (Mustaffa-Babjee et al., 1974; Nerome et al., 1978).

Similar to IAVs, detection of APMVs in both poultry and wild birds has traditionally relied on virus isolation in specific-pathogen free embryonated chicken eggs followed by hemagglutination assay on allantoic fluid and RT-PCR to identify the virus present (Alexander and Senne, 2008). With advancements in molecular methods, labs can now quickly detect NDV RNA in clinical samples using real time RT-PCR targeting the matrix gene (Wise et al., 2004; Hines et al., 2012). However, detecting the presence of other APMVs relies on using RT-PCR to detect viral RNA to each subtype specifically and/or using hemagglutination inhibition assay and anti-sera from each of the APMVs which has greatly hindered large-scale studies (Alexander and Senne, 2008). However, recently, a new set of universal Paramyxoviridae family primers was described which are reported to amplify a 121 bp fragment of the polymerase gene for all tested AMPVs (van Boheemen et al., 2012). This protocol decreases the time needed to determine if allantoic fluid contains RNA from a virus in the Paramyxoviridae family; however, post RT-PCR processing (sequencing) or using another assay, such as the hemagglutination inhibition assay, is needed to determine what subtype was isolated.

Avian Paramyxoviruses in Wild Birds

What is known about APMVs in wild birds is in large part due to research on IAVs because isolation methods for IAVs will also isolate APMVs (see above). Avian paramyxoviruses have been reported from several waterfowl species from various locations in the United States (Hinshaw et al., 1985; Nettles et al., 1985; Stallknecht et al., 1991; Hanson et al., 2005; Goekjian et al., 2011). Much like IAVs in waterfowl, APMVs are not associated with

any clinical disease, even in experimentally infected ducklings (Kim et al., 2012b). Among waterfowl in North America the most common APMVs isolated have been NDV, APMV-2, APMV-4, APMV-6, and APMV-8 with APMV-3 and APMV-7 being isolated infrequently (Kocan et al., 1979; Alexander, 1980; Vickers and Hanson, 1982; Hinshaw et al., 1985; Stallknecht et al., 1991; Hanson et al., 2005). Unlike IAVs, there does not appear to be a clear temporal or spatial distribution in the circulation of APMVs with viruses being isolated in >10% of birds sampled on wintering grounds (Hanson et al. 2005). Although, one study reported peak viral shedding of NDV in wood ducks occurred in late July and early August in North Carolina (Goekjian et al. 2011). It has not been thoroughly investigated, but transmission of APMVs in waterfowl is believed to be by the fecal-oral route, because the virus is readily isolated from cloacal swabs and ducks are able to become infected by oral inoculation (Vickers and Hanson 1982; Hanson et al. 2005). Outside of waterfowl disease has been documented with infection of NDV. Epornitics from NDV have been documented in double-crested cormorants (*Phalacrocorax auritus*) (Kuiken et al., 1998; Glaser et al., 1999).

Serology of APMVs in wild birds has been poorly studied and because of this very little information is known about exposure to these viruses. Only two studies have been conducted on ducks in North America. These studies only looked for antibodies to NDV and detected prevalence <16% in several waterfowl species (Kocan et al., 1979; Vickers and Hanson, 1982). Another study found NDV antibody prevalence in >90% of double-crested cormorants in nesting colonies in Michigan (Cross et al., 2013). Outside of North America two studies have analyzed exposure of waterfowl to APMVs. One study conducted in Spain found antibodies to NDV, APMV-2, and APMV-3 (Maldonado et al., 1995). The other study in New Zealand found antibodies to NDV and APMV2-APMV9 in mallards (Stanislawek et al., 2002).

Research on APMVs other than NDV is largely surveillance studies in wild birds. Other than APMV-2, APMV-3, and APMV-5 there has not been any disease associated with these viruses (Bradshaw and Jensen, 1978; Nerome et al., 1978; Tumova et al., 1979). One study has done serologic screening for nine APMV serotypes in poultry and detected antibodies to serotypes 1-9 with NDV having the highest titers (Warke et al., 2008a). Experimentally, mild disease has been seen in chickens challenged with APMV-3 and APMV-4 (Warke et al., 2008b; Nayak et al., 2012). In experimentally infected ducks no disease was reported after challenge with NDV and APMV-2-APMV-9 (Kim et al., 2012b). This study found no evidence of APMV-5 replication in ducks.

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CHAPTER 3

SPATIAL DISTRIBUTION OF INFLUENZA A VIRUSES IN POST-BREEDING CANADA GEESE (*Branta candadensis*) IN PENNSYLVANIA

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Abstract: Canada geese have a near ubiquitous distribution and share aquatic habitats with influenza A virus (IAV) reservoir species. Although they were thought to play a limited role in the transmission of (IAVs) in wild birds, recent serologic surveys for IAVs in Canada geese showed they are frequently exposed to IAVs. The objective of this study is to determine prevalence and annual variation of IAVs antibodies in Canada geese and evaluate if there is a spatial pattern to IAVs antibodies distribution in Canada geese in Pennsylvania. We sampled 2,919 geese from 115 locations from 2009-2012 and IAV antibodies were detected using a commercial blocking ELISA. Logistic regression models with generalized estimating equations and predictor variables were used to understand risk factors associated with exposure to IAVs. Overall, there was no significant variation of antibody prevalence among the four years of this study and we only detected a significant difference at one location out of 12 that were sampled in multiple years. The percent developed land was the only significant predictor for IAV exposure in geese. The lack of annual variation in antibody prevalence indicates that serology to the IAV nucleoprotein may not have enough definition to detect yearly variation in IAV circulation, which has been documented with virus isolation in dabbling ducks. In addition, our spatial modeling indicates that serology may not be sensitive enough to detect areas of increased risk of transmission for IAVs. Canada geese utilizing urban habitats have increased survival compared to geese utilizing rural habitats and coupled with antibody duration of >1-year likely explains the increased risk of exposure in these areas. In conclusion, serology in Canada geese is likely not able to detect subtle changes in antibody prevalence on a small (state-wide) spatial scale.

Keywords: Canada geese, Influenza A Viruses, Serology, Spatial Modeling

Introduction

Wild birds in the orders Anseriformes and Charadriiformes are considered the natural reservoirs for influenza A viruses (IAVs) (Olsen et al., 2006). Traditional surveillance for IAVs in wild birds has relied on viral detection by either virus isolation or RT-PCR (Hinshaw et al., 1985; Wallensten et al., 2007). However, over the last decade there has been an increase in the use of serologic assays for IAVs surveillance in wild birds (Brown et al., 2010a, 2010b). Serologic assays have benefits over virus detection assays because the duration of detectable antibodies can be >1 year in naturally infected birds (Fereidouni et al., 2010; Tolf et al., 2013) and viral shedding may only be detected for <1-month (Costa et al., 2011). This allows for sampling during time when birds are easier to sample (e.g. during molting), which may not correspond IAV circulation.

Canada geese (*Branta canadensis*) are experimentally susceptible to infection with IAVs and have a near ubiquitous distribution in the United States (US), which overlaps with known IAVs reservoir species (Hestbeck, 1995). However, IAV isolations from Canada geese are rare (Harris et al., 2010). This low detection of viral shedding may be related to a viral shedding pattern that is sporadic and of short duration (Berhane et al., 2014; Pasick et al., 2007) or sampling during periods when IAV circulation is rare (Harris et al., 2010; Kistler et al., 2012). Recently, serologic testing showed that Canada geese are frequently exposed to IAVs despite the infrequent isolation of IAVs from this species (Kistler et al., 2012).

Understanding environmental risk factors associated with transmission and/or exposure of IAVs can help direct future research on sampling strategies. Previous research in North America has identified temperature, percent harvested cropland, and previous isolation of IAVs in a wetland increasing the risk of subsequent IAV isolations in wild birds (Farnsworth et al.,

2012; Fuller et al., 2010). In addition, a spatial pattern of IAV antibody prevalence in Canada geese has been shown on a regional scale (Kistler et al., 2012). The objectives of this study are to determine prevalence and annual variation of IAVs antibodies in Canada geese and to evaluate if there is a spatial pattern associated with IAVs antibody prevalence on a local (state) scale.

Materials and Methods

In June and July from 2009-2012, we collected blood samples (n=2,919) from Canada geese from 115 locations in Pennsylvania during bird banding and nuisance removal programs. Additional samples were included from a recent study (Kistler et al., 2012). Sample collections occurred during banding efforts with the Pennsylvania Game Commission and nuisance removal programs conducted by the United States Department of Agriculture Wildlife Services. Blood samples were collected from the medial metatarsal vein from geese being released and by cardiocentesis from birds that were euthanized. After collection blood samples were placed in Vacutainer® serum separator tubes (BD, Franklin Lakes, NJ, USA) and placed on wet ice in the field. After transport to a laboratory (<1 day) blood samples were centrifuged (15 min at 1200g) and serum was removed and stored at -20C until testing. We determined prevalence of antibodies to the IAV nucleoprotein using a commercial blocking enzyme-linked immunosorbent assay (IDEXX Laboratories, Westbrook, ME, USA) following the manufacturer's protocol (Brown et al., 2009).

We collected GPS data from each sample location and used a circular buffer with an area of 138 hectares to estimate post breeding Canada goose home range (Dunton and Combs, 2010). We calculated the percent coverage of home range by four environmental variables (Table 1) within this buffer. Developed area combined all four developed land categories from the 2011

National Land Cover Database (NLCD) map. Cropland was derived for each year of the study from the Cropland Data Layer (CDL) and mapped grain crops, corn, and sunflowers; crops commonly fed on by geese (Gates et al., 2001). Grassland data (hay, clover, and switchgrass) also was mapped from CDL. All raster datasets were resampled to a 60km. National Wetland Inventory (NWI) polygon was used to depict available wetland habitat and converted to a raster with 60m resolution. Zonal statistics were used to calculate area of each raster layer covered by sample home range. We included ecoregions from sample locations as a binary variable to investigate the association between IAV antibodies and spatial patterns. We used ecoregions because they encompass several environmental variables (e.g. temperature, vegetation, etc.) that are correlated, including climatic variables that were found important risk factors for IAV circulation (Farnsworth et al., 2012; Fuller et al., 2010).

All spatial analysis was conducted in ArcMap v10.2 (ESRI, Redlands, CA, USA) and logistic regression models utilized generalized estimating equations were implemented in R v3.0 (http://cran.us.r-project.org/) using the Zelig package (Lam, 2014). Logistic regression utilizing generalized estimating equations (GEE) was used to account for correlation of multiple samples coming from the same location at the same time. We ran a model for all four years of sampling and used Wald χ^2 test was used to determine significance of predictor variables. Moran's I was calculated on the model residuals to determine spatial autocorrelation of the residuals using the *spdep* package in R. This was done using first order up to fifth order nearest neighbors. We used logistic regression utilizing GEE to compare results across years and to compare results from locations that were sampled in multiple years.

Results

The bELISA test results for all four years are summarized in Table 2. In total, we collected samples from 115 sites across Pennsylvania (Figure 1). We detected an overall antibody prevalence of 24% (714/2,919) and we did not detect a significant difference among years (Table 2). We sampled 12 locations in multiple years with nine of the locations being sampled in two years and three locations being sampled in three years; at only one of these locations did we detect a significantly higher antibody prevalence in 2011 compared to 2009 (OR 2.5, 95% CI 1.3 – 4.8). At this particular site the birds were euthanized every year and a new flock moved into the area making their exposure history different than the previous flock.

We used the samples from all four years in our spatial model. We did not detect any differences in IAV antibody prevalence among the Pennsylvania ecoregions. Additionally, percent of home range covered by wetlands, cropland, and grassland were not significantly associated with IAV exposure of Canada geese. Only higher percentage of home range cover being developed area was associated with increased risk of exposure in geese (estimate= 0.0111; Wald χ^2 =12.2; p-value<0.005). The odds of a goose flock being exposed to IAVs increased 74% for every 5% increase in developed land coverage. The Moran's I statistic for model residuals was .0186 (p-value=0.6; Figure 3.2), indicating we did not have unaccounted spatial pattern in the model residuals.

Discussion

We only detected minimal variations of IAVs antibody prevalence in geese among years; however, these differences were not significant. Additionally, 11 of 12 locations that were sampled in multiple years showed no significant variation among years. These results support

previous research showing little annual variation in IAV antibody prevalence in goose species (Kistler et al., 2012; Wilson et al., 2013) which suggests that NP antibody prevalence is not sensitive enough to detect annual variation in transmission pattern of IAVs seen in dabbling ducks with virus isolation (Hinshaw et al., 1985). This is likely due to the persistence of the antibody response (Tolf et al., 2013), seasonal movements of resident geese, and possibly the age structure of these populations. With regard to movements, resident geese can undergo seasonal migrations >200km (Dieter et al., 2010) and infection may have occurred away from their post-breeding grounds where they were sampled in the present study. Our antibody prevalence is lower than that reported in goose species in Alaska and Europe (Hoye et al., 2011; Wilson et al., 2013). This lower antibody prevalence may be associated with decreased exposure from IAVs circulating in dabbling ducks in Pennsylvania. Kistler et al., (2012) found that geese in higher latitudes had increased antibody prevalence than those in lower latitudes. These higher latitudes correspond with the prairie pothole region of North America where dabbling duck production and IAV shedding prevalence is higher than in southern locations (Hinshaw et al., 1985; Stallknecht et al., 1990).

We used a logistic regression model to estimate risk factors for exposure of Canada geese to IAVs. This is the first attempt to use serologic data to evaluate environmental risk associated with IAVs in wild birds. Unlike two previous studies, we did not find temperature to be a significant predictor for IAVs exposure (Farnsworth et al., 2012; Fuller et al., 2010); however, we only used ecoregions as a proxy for temperature variables and these may not be sensitive enough to detect differences in temperature. Alternatively, we sampled across a limited geographic region where fluctuation in temperature and climate is not as noticeable, whereas the other two studies sampled across the contiguous United States. In addition, we did not find %

cropland to be a significant risk for exposure of IAVs in geese. In Asia, cropland has been frequently associated with highly pathogenic H5N1 outbreaks in wild birds and poultry; however, rice is the main crop associated with these outbreaks (Gilbert et al., 2008). Rice, unlike most crops cultivated in the US, is cultivated in water which is an important medium for facilitating the transmission of IAVs (Breban et al., 2009) and this probably explains the lack of increased risk associated with % cropland in our study.

The only significant predictor variable we found affecting IAV exposure in post-breeding Canada geese was % of developed land within their estimated home range. This increased risk of exposure is probably not associated with increased transmission IAVs in these areas, but is likely due to increased survival of geese in urban areas and persistence of IAV antibodies. Although not survival in different habitats has not been evaluated in Pennsylvania, urban Canada geese in Georgia had significantly higher survival rates and significantly lower hunter mortality than those in a rural area (Balkcom, 2010). This combined with persistence of IAV antibodies for >1 year in naturally infected birds even in the absence of exposure to virus (Fereidouni et al., 2010; Tolf et al., 2013), likely explains this increased risk of exposure.

Although serology has been successfully used to map risk factors associated other pathogens, e.g., pseudorabies virus in European wild boars (*Sus scrofa scrofa*), it is likely not sensitive enough to detect areas of increased risk of transmission of IAVs in wild birds, even resident species, at a state-level scale. In conclusion, serologic assays are a valuable tool in understanding the epidemiology of IAVs in wild birds; however, the interpretation of serologic data has limitations when applied on small spatial scales.

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Table 3.1. Description and sources for predictor variables for predicting risk of exposure to influenza A viruses in Canada geese.

Variable	Variable description	Units	Range	Source
Developed Land Cover	Percent wetland cover of home-range	% Cover	0-99.8	National Land Cover Data Base 2011 United States Geological Survey (USGS): http://www.mrlc.gov/
Wetland Cover	Percent wetland cover of home-range	% Cover	0-80.9	National Wetland Inventory United States Fish and Wildlife Service
Grassland	Percent of home-range covered. Done for each year	% Cover	0-42	Cropland Data Layer, National Agriculture Statistics Service United States Department of

				Agriculture http://www.nass.usda.gov/research/Cropland/SARS1a.htm
Cropland	Percent of home-range covered. Done for each year	% Cover	0-61.5	Cropland Data Layer
Ecoregions	Sample location in ecoregion	Presence	0 or 1	United States Environmental Protection Agency

Table 3.2. Prevalence estimates to influenza A virus nucleoprotein as determined by bELISA. Odds ratios and 95% confidence intervals from logistic regression utilizing generalized estimating equations.

Year (Locations)	Positive/Sampled (%)	Odds Ratio (95% CI)
2009* (28)	140/694 (20)	Referent
2010 (23)	137/495 (28)	1.6 (0.9-2.6)
2011 (37)	242/861 (28)	1.6 (0.9-2.6)
2012 (27)	195/869 (22)	1.1 (0.6-2.0)
Total (115)	714/2,919 (24)	

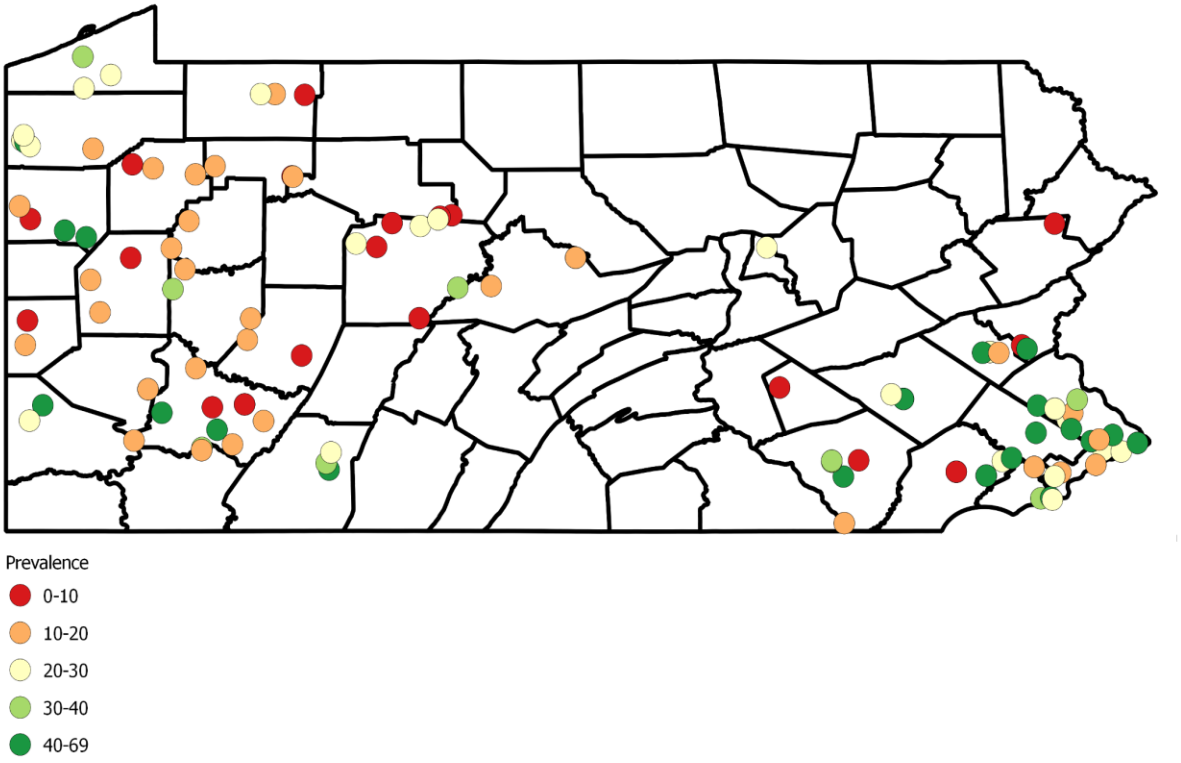

Figure 3.1. Influenza A virus nucleoprotein antibody prevalence in Canada geese as determined by bELISA by sample location from 2009-2012.

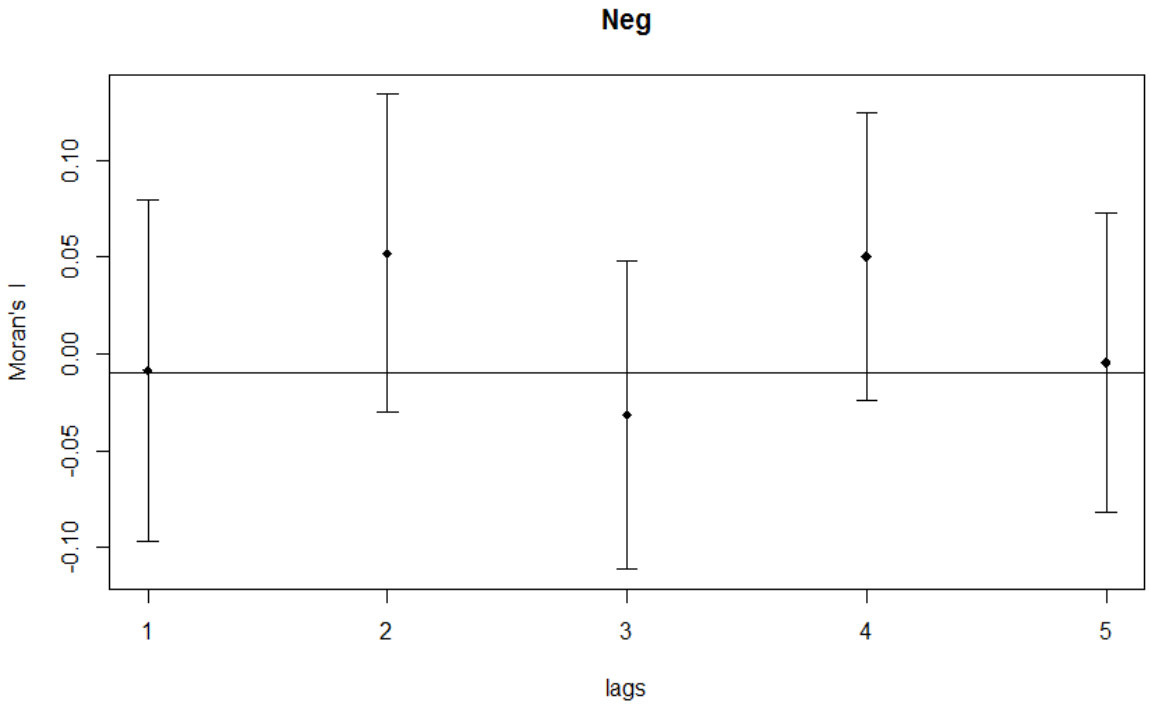

Figure 3.2. Plot of Moran's I values from first order nearest neighbors to fifth order nearest neighbors.

CHAPTER 4

SUBTYPE-SPECIFIC INFLUENZA A VIRUS ANTIBODIES IN CANADA GEESE (*Branta canadensis*)

W. M. Kistler, D. E. Stallknecht, T. J. DeLiberto, K. Van Why, and M. J. Yabsley. To be submitted to *BMC Veterinary Research*

Abstract: Traditionally, surveillance for influenza A viruses (IAVs) in wild birds has relied on viral detection assays. However, serological assays have been recently developed that have increased our understanding of IAVs in wild birds. Serological surveillance for IAV antibodies in Canada geese (*Branta canadensis*) has shown that, despite a low prevalence of virus isolations, Canada geese are frequently exposed to IAVs and that exposure increases with latitude, which follows virus isolation prevalence patterns observed in dabbling ducks. The objectives of this study were to further evaluate IAV antibodies in Canada geese using a subtype-specific serological assay to determine if Canada geese are exposed to subtypes that commonly circulate in dabbling ducks. We collected serum samples from Canada geese in Minnesota, New Jersey, Pennsylvania, and Wisconsin and tested for antibodies to the IAV nucleoprotein (NP). Positive samples were further tested by hemagglutination inhibition for 10 hemagglutinin IAV subtypes (H1-H10). Overall, we detected antibodies to NP in 24% (714/2,919) of geese. Antibodies to H3, H4, H5, and H6 subtypes predominated, with H5 being detected most frequently. A decrease in H5 HI antibody prevalence and titers was observed from 2009 to 2010-2012. We also detected similar exposure pattern in Canada geese from New Jersey, Minnesota, Washington and Wisconsin. Based on the published literature, H3, H4, and H6 viruses are the most commonly reported IAVs from dabbling ducks. These results indicate that Canada geese also are frequently exposed to viruses of the same HA subtypes; however, the high prevalence of antibodies to H5 viruses was not expected as H5 IAVs are generally not well represented in reported isolates from ducks.

Keywords: Canada geese, Hemagglutination Inhibition, Influenza A Virus, Sentinel, Serology

Introduction

Wild birds in the orders Anseriformes and Charadriiformes are considered the natural reservoirs for influenza A viruses (IAVs) (Oslen et al., 2006) and traditional surveillance for these viruses in wild birds has relied on viral detection by either virus isolation or RT-PCR (Hinshaw et al., 1985; Wallensten et al., 2007). However, serological assays have been recently developed that reportedly have a high sensitivity at detecting antibodies to IAVs, thus these assays can be used to improve surveillance approaches (Brown et al., 2009; Lebarbenchon et al., 2012). The duration of detectable antibodies can be >1 year in naturally infected ducks (Tolf et al., 2013; Feriedouni et al., 2010), and with repeated infections, they may persists for the life of the bird. In contrast, viral shedding is of short duration, often <10 days (Pasick et al., 2007; Costa et al., 2011). The long duration of antibodies allows for sampling during times when birds are more easily captured (e.g. summer molting) or in species where information about their role in the maintenance of IAVs is limited. Serology has been recently used to supplement virus isolation data and advance our current understanding of IAVs in Canada geese (*Branta canadensis*) (Kistler et al., 2012).

Traditionally, Canada geese have not been implicated in an important role in the epidemiology of IAVs. Although Canada geese have a near ubiquitous distribution in the United States (US) and share aquatic habitats with known IAVs reservoir species (Hestbeck, 1995), IAV isolations from Canada geese are rare (Harris et al., 2012). This perceived low prevalence of viral isolation is likely due to brief and infrequent viral shedding patterns reported in experimentally infected Canada geese (Berhane et al., 2014) and sample timing which often occurred during a 3-4-week flight-less molting period during June and early July (Harris et al., 2010; Kistler et al., 2012). Using serologic testing, Canada geese were found to be frequently

exposed to IAVs and the prevalence of antibodies increased with latitude (Kistler et al., 2012). This increase in antibody prevalence in geese followed a similar trend of virus shedding data in dabbling ducks (Hinshaw et al., 1985; Stallknecht et al., 1990).

These data suggest that serological surveillance of IAVs in Canada geese may provide an inexpensive sentinel system to monitor or supplement surveillance efforts to understand spatial and annual trends in IAV transmission in waterfowl populations. However, subtype-specific serological data are needed to understand if antibodies detected in Canada geese are representative of the predominant subtypes detected in waterfowl, especially dabbling ducks. Based on virus isolation results from dabbling ducks, hemagglutinin subtypes H3, H4, and H6 are most commonly reported during peak IAV transmission in late summer and early fall (Wilcox et al., 2011). The objectives of this study were to determine long term trends in IAVs antibodies to the nucleoprotein (NP) and to detect subtype-specific antibodies in Canada geese.

Materials and Methods

In June and July 2010-2012, we collected blood samples from Canada geese from 82 locations (Figure 1) in Pennsylvania during banding and nuisance removal programs. Blood samples were collected from the medial metatarsal vein from geese being released and by cardiocentesis from birds that were euthanized. Blood samples were placed in Vacutainer® serum separator tubes (BD, Franklin Lakes, NJ, USA) and placed on wet ice in the field. After transport to a laboratory (<1 day) blood samples were centrifuged (15 min at 1200g) and serum was removed and stored at -20C until testing. Canada goose samples collected in 2009 during a previous study (Kistler et al., 2012) were also used in this study. These 2009 samples had antibodies to IAV NP and were from New Jersey (n=63), Minnesota (n=14), Pennsylvania

(n=134), and Washington (n=26). In addition, samples (n=80) with IAVs NP antibodies collected from 11 locations in Wisconsin during 2010 and 2011 were tested. All techniques were reviewed and approved by the IACUC committee at the University of Georgia.

We first screened serum samples for presence of antibodies to the IAV NP using a commercial blocking enzyme-linked immunosorbent assay (bELISA; IDEXX Laboratories, Westbrook, ME, USA). Samples that had antibodies to the IAV NP were then screened by a hemagglutination inhibition (HI) assay using antigen from the Southeastern Cooperative Wildlife Disease Study (University of Georgia, Athens, GA, USA; Table 1) and positive control serum from specific pathogen-free chickens (National Veterinary Service Laboratories, United States Department of Agriculture, Ames, IA, USA). Canada goose serum was first treated with 10% chicken red blood cells (1:1 dilution), incubated at room temperature for 1 hour, and then centrifuged for 10 min at 800g. The supernatant was then removed and used for the HI assays. The HI assays for all subtypes were conducted as previously described (Hinshwa et al., 1986) using 4 HA/25μl and a positive cut-off titer of $\geq$32.

Results

Overall, we detected antibodies to the IAV nucleoprotein in 24% (714/2,919) of Canada geese in Pennsylvania. We collected samples from 31 counties in Pennsylvania. Nucleoprotein antibody prevalence was highest in the northwestern and southeastern counties (Figure 1). No significant difference in antibody prevalence was detected among years (Table 2). Of the 714 samples with NP antibodies, we used 653 samples for subtype-specific antibody testing. Antibodies to the H5 subtype were detected most often with 60% of the NP-positive birds testing positive. There was a decrease in both H5-specific antibody prevalence and geometric mean titer

from 2009 through 2012 (Table 3). Antibodies to the H3, H4, and H6 subtypes were also detected in >20% of NP-positive geese, but there was little fluctuation in antibody prevalence across years (Figure 2). Within Pennsylvania, we detected H5-specific antibodies from 88% (102/116), H3-specific antibodies from 63% (73/116), H4-specific antibodies from 59% (69/116), and H6-specific antibodies from 71% (82/116) of sample location. There was also little fluctuation in antibody prevalence to H7 and H9 subtypes (Figure 2). Antibody prevalence to the remaining four subtypes (H1, H2, H8 and H10) was estimated at <1%.

To further evaluate if subtype-specific antibodies were consistent on a larger scale, we tested an additional 183 samples from four other states for antibodies to H3, H4, H5, and H6 subtypes. Antibodies to the H5 subtype also were the most frequently in these states. While antibodies to the H3 and H6 subtypes were >20%, H4-specific antibodies were <10%. We did not detect any H4-specific antibodies from Wisconsin in 2011.

Discussion

This study was a continuation of previous serological survey conducted in Canada geese (Kistler et al., 2012). We detected slight variations of IAVs nucleoprotein antibody prevalence among years; however, these changes were not significant. In a previous study, antibody prevalence estimates from Canada geese were shown to increase with latitude (Kistler et al., 2012), which corresponds with IAV isolation data from dabbling ducks (Hinshaw et al., 1985; Stallknecht et al., 1990). These data suggested that Canada geese share a common exposure to IAVs with dabbling ducks. Although IAVs from all avian HA subtypes, except for the H16 subtype, have been detected from dabbling ducks, the H3, H4, and H6 subtypes are the most frequently isolated subtypes (Wilcox et al., 2011). In our study, antibodies to H3, H4, and H6

were frequently detected which supports a common source of exposure in Canada geese. This source is likely through direct contact of dabbling ducks or through the environment contaminated with IAVs. The low prevalence of H1, H2, H8, and H10 was not surprising as these subtypes are often under-represented in IAVs detected in dabbling ducks (Sharp et al., 1993).

Influenza A viruses of the H5 subtype are usually detected in low prevalence from dabbling ducks and often account for <1% of detected IAVs (Hanson et al., 2008; Wilcox et al., 2011). However, local variation in the prevalence of H5 IAVs in ducks has been reported (Lindsay et al., 2013). The high H5 antibody prevalence we detected in Canada geese is not understood, but could be related to this subtype circulating outside the known peak viral shedding in dabbling ducks when most sampling occurs. A higher prevalence of H5 IAVs has been detected in ducks in the winter months in California (Pearce et al., 2011; Hill et al., 2012). If the transmission of H5 viruses is dependent on season and more likely to occur during winter, Canada geese would be exposed to these viruses closer to our sampling time. Alternatively, the high H5-specific antibody prevalence we detected could be due to a more robust immune response associated with the H5 subtype as Canada geese experimentally infected with H5 viruses develop higher subtype specific antibody titers than when infected with other subtypes (Berhane et al., 2010). These higher titers are likely due to H5 viruses replicating more efficiently in geese than other subtypes. In experimental infections, geese shed virus and develop antibodies after a single inoculation with H5 viruses, but often need to be inoculated more than once with other subtypes for viral shedding and a detectable antibody response (Berhane et al., 2010; 2014). The high H5 antibody prevalence we detected across multiple states and locations indicates this subtype circulates across the country.

We detected a decrease in H5-specific antibody titer and prevalence every year in Pennsylvania from a peak in 2009. This decrease in antibody titer and prevalence is likely due to a decrease in exposure to this subtype, population recruitment, and a waning subtype-specific antibody response. Annual variation in HA subtype prevalence has been reported in dabbling ducks (Hinshwaw et al., 1985; Krauss et al., 2004). Although there is very little information on the persistence of subtype-specific antibodies in wild birds, antibodies to the IAV nucleoprotein have been shown to persist for 1-year in the absence of detected virus circulation (Tolf et al., 2013). The observed variation could also have resulted from our use of one H5 antigen for all assays, regardless of year. The extent of H5 antigenic diversity in North American IAVs is not known but any antigenic changes could have altered our prevalence and titers detected. Although antigenic drift has been associated with new strains of highly pathogenic H5N1 viruses Zhone et al., 2014) it is poorly documented with low pathogenic H5 viruses from wild birds.

We detected similar subtype-specific antibody prevalence estimates in Canada geese sampled outside of Pennsylvania. Although, we only tested for antibodies to four HA subtypes, geese from these states had similar proportion of antibodies to these subtypes with antibodies to the H5 subtype predominating; however, we did not detect any antibodies to the H4 subtype in Wisconsin in 2011. This may be likely to low circulation of H4 IAVs during that year. In different locations and years there is variation in subtypes that circulate in wild ducks (Wilcox et al., 2011).

Overall, we detected little variation in IAV antibody prevalence in Canada geese from Pennsylvania. In addition, geese were frequently exposed to subtypes that commonly circulate in dabbling ducks. We detected a high prevalence of H5-specific antibodies and these viruses are not well represented among viruses isolated from dabbling ducks. The H5-specific antibody

prevalence could be due to increased antibody response to this subtype in Canada geese or circulation of this subtype after peak viral shedding is seen in dabbling ducks. These results support that Canada geese can be used as a serologic sentinel for IAV distribution on a regional scale; however, additional information related to antibody response in this species and seasonal variation in subtype prevalence are needed to fully interpret serologic data.

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Table 4.1. Viruses used to make antigen for the hemagglutination inhibition assay.

Hemagglutinin Subtype	Virus
H1	A/Mallard/Minnesota/sg-00627/2008(H1N1)
H2	A/Mallard/Minnesota/AI08-2755/2008(H2N3)
H3	A/Mallard/Minnesota/Sg-00627/2008(H3N8)
H4	A/Mallard/Minnesota/Sg-01049/2008(H4N6)
H5	A/Mallard/Minnesota/AI08-3532/2008(H5N2)
H6	A/Mallard/Minnesota/Sg-00796/2008(H6N1)
H7	A/Mallard/Minnesota/AI09-3770/2009(H7N9)
H8	A/Mallard/Minnesota/Sg-00689/2008(H8N4)
H9	A/Mallard/Arkansas/AI09-5649/2009(H9N2)
H10	A/Mallard/Minnesota/Sg-00689/2008(H10N7)

Table 4.2. H5-specific antibody titers and prevalence across years as determined by the hemagglutination inhibition assay.

Year	Positive/Sampled (%; 95% CI)	Geometric Mean Titer
2009	127/134 (95; 91-99)	163
2010	101/113 (89; 84-95)	130
2011	125/222 (56; 48-65)	37
2012	42/184 (23; 16-30)	33

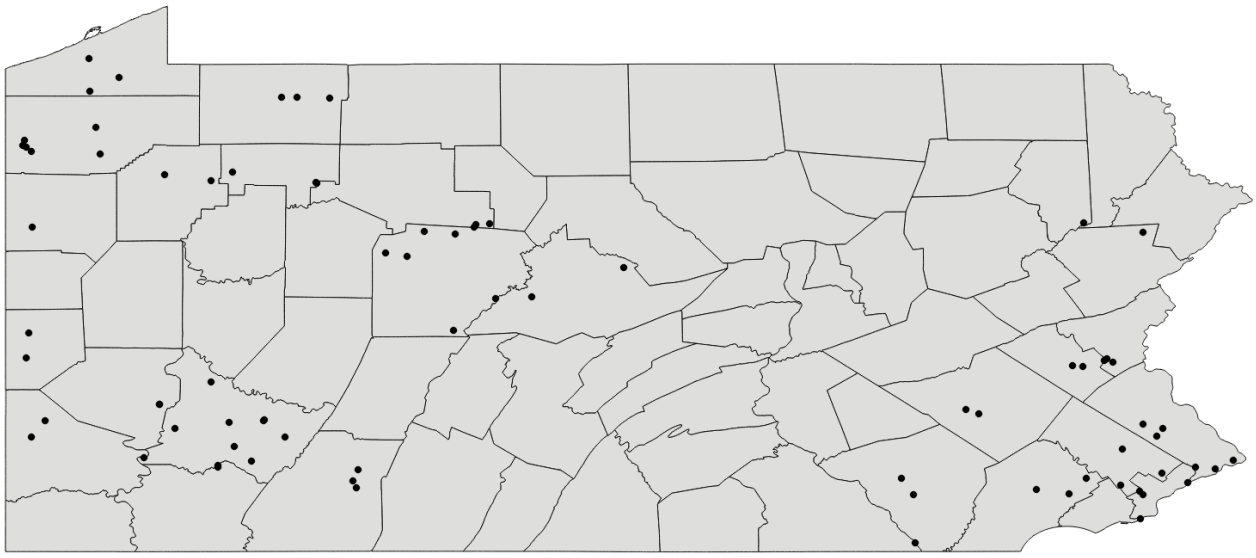

Figure 4.1. Sample location distribution in Pennsylvania 2010-2012.

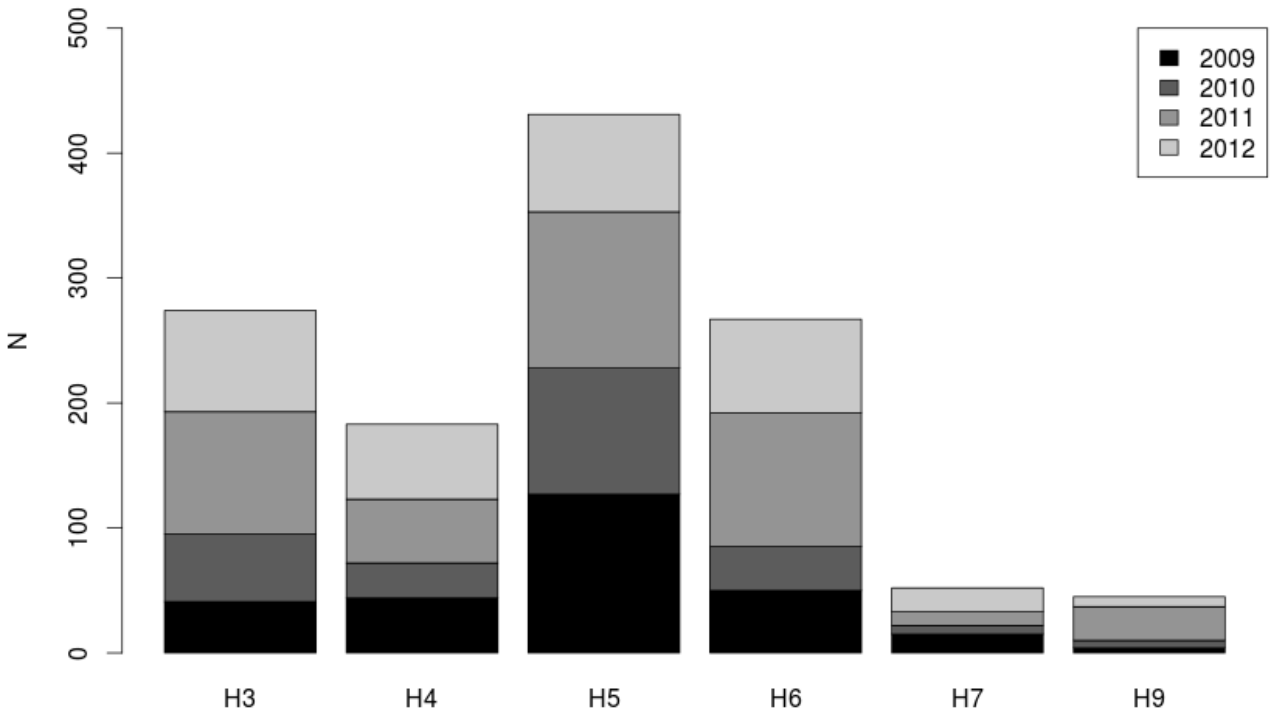

Figure 4.2. Influenza A virus subtype-specific antibodies from (n=653) Canada geese in Pennsylvania as determined by the hemagglutination inhibition assay.

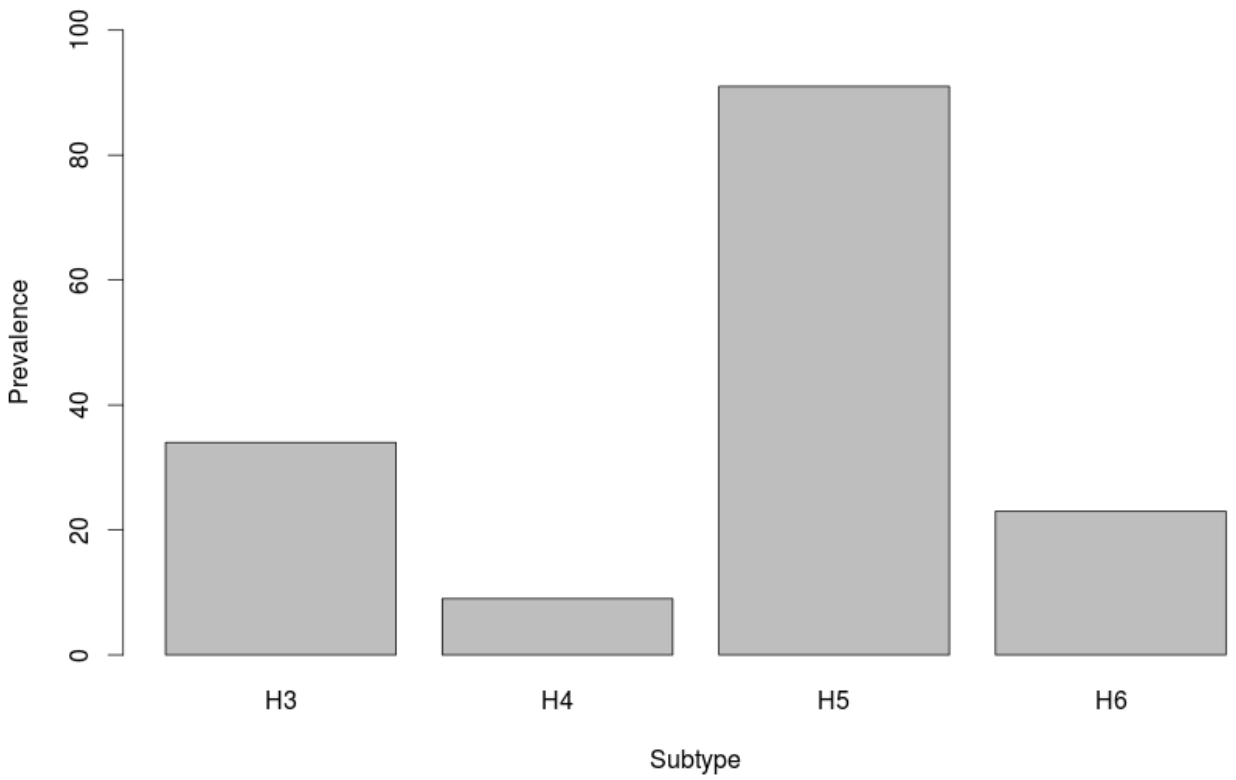

Figure 4.3. Prevalence of Influenza A subtype-specific antibodies from (n=184) Canada geese in New Jersey, Minnesota, Washington, and Wisconsin as determined by the hemagglutination inhibition assay.

CHAPTER 5

INFLUENZA A VIRUS H5-SPECIFIC ANTIBODIES IN NORTH AMERICAN MUTE SWANS (*Cygnus olor*)

W. M. Kistler, D. E. Stallknecht, C. Lebarbenchon, K.Pedersen, D. R. Marks, R. Mickley, Thomas J. DeLiberto, and M. J. Yabsley. To be submitted to the *Journal of Wildlife Diseases*.

Abstract: The use of serological assays for influenza A virus (IAV) surveillance in wild birds has increased due to the availability of commercial enzyme-linked immunosorbent assays (ELISAs). Recently, an H5-specific blocking ELISA was shown to reliably detect H5-specific antibodies to low and high pathogenic H5 viruses in experimentally-infected waterfowl. Mute swans (*Cygnus olor*) are large conspicuous birds that were frequently associated with highly pathogenic H5N1 outbreaks in Europe. Because mute swans are present in North America and may play a similar role if highly pathogenic H5N1 were introduced into North America, the objective of this study was to determine the NP and H5 IAV antibody prevalence in mute swans using multiple serological assays. We collected 340 serum samples from mute swans in Michigan, New Jersey, New York, and Rhode Island. In total, we detected antibodies to the IAV NP in 66% (225/340) of the samples. We detected H5-specific antibodies in 63% (214/340) and 19% (64/340) using a modified bELISA protocol and HI assay, respectively. In comparing the two H5 bELISA protocols, the modified protocol detected significantly more positive samples than the manufacturer's protocol (McNemar's $\chi 2=63$, p-value<0.001); although there was fair agreement between the protocols ($\kappa =0.3$ (0.1-0.5). We also tested 46 samples with a virus neutralization assay. This assay had high agreement with the second bELISA protocol and detected a higher prevalence then the HI assay. Overall, these results indicate that North American mute swans have a high H5 antibody prevalence and may be protected from disease associated with highly pathogenic H5N1.

Keywords: H5-specific ELISA, hemagglutination inhibition, mute swans, serology

Surveillance for influenza A viruses (IAVs) in wild birds has been traditionally based on detection of viral shedding using virus isolation or polymerase chain reaction. However,

recently, there has been an increase in the use of serological assays for IAV surveillance because of the development and validation of several commercial availability enzyme-linked immunosorbent assays (ELISA) in several wild bird species (Brown et al., 2009). These assays detect antibodies to the IAV nucleoprotein (NP) and have been effectively used for IAV surveillance in wild birds (Brown et al., 2010; Kistler et al., 2012). However, the commercial assays that have been validated for use in wild birds detect antibodies to all IAVs and are not capable of detecting subtype specific antibodies.

Historically, the hemagglutination inhibition (HI) assay has been the most frequently to detect subtype specific antibodies in wild birds (Winkler et al., 1972). The HI assay can detect IAV antibodies across multiple species and has been used in large scale surveillance studies in wild birds (De Marco et al., 2004; Niqueux et al., 2010). However, there are several disadvantages to the HI assay, the most important of which is a lack of standardization with antigen and antisera across laboratories making it difficult to compare results among studies (Stephenson et al., 2007). The recent development of a blocking ELISA that has high sensitivity and specificity for detection of H5-specific antibodies in experimentally infected mallards (*Anas platyrhynchos*; Lebarbenchon et al., 2013) offers a possible assay that can provide standardized results across laboratories.

The emergence of highly pathogenic (HP) H5N1 in poultry in 1997 has increased the availability of H5-specific antibody tests. Over the last 15 years, HP H5N1 viruses have been reported in wild birds across Asia and Europe, and in particular, mute swans (Cygnus olor) where frequently associated with outbreaks of HP H5N1 in Europe (Feare, 2010). Mute swans are large conspicuous birds and may play a role if HP H5N1 were to be introduced to North

America. The objective of this study was to determine if H5 IAV antibodies currently exists in North American mute swans using several H5-specific serologic assays.

Blood samples were collected from mute swans (n=340) in collaboration with the United States Department of Agriculture-Wildlife Services during nuisance collections in Michigan, New Jersey, New York, and Rhode Island. Blood samples were collected by cardiocentesis from birds being euthanized or the medial metatarsel vein from birds being released. Blood samples were placed in vacutainer® tubes (BD, Franklin Lakes, New Jersey) stored on wet ice in the field until transport to a laboratory (<24 hours). Samples where then centrifuged for 10min at 3000rpm and sera was stored at -20ºC until testing.

Antibodies to the all IAV serotypes were detected using a commercial NP bELISA (IDEXX Laboratories, Westbrook, ME) per manufacturer's instructions (Brown et al., 2009). We detected H5-specific antibodies using three assays; a bELISA (ID VET, Montpelier, France), an H5 HI assay, and an H5 virus microneutralization assay (VN). We used two different protocols with the H5-specific bELISA, 1) we followed the manufacturer's instructions and 2) we used a 1:2 serum dilution with an 18 hour incubation at 36ºC as modified and described by Lebarbenchon et al., (2013). We tested a subset of 182 serum samples with both bELISA protocols and the remaining 158 with the modified protocol. Samples with sample to negative ratios >0.35 were considered negative. We tested all 340 serum samples by HI using A/mallard/AI08-3532/H5N2 as antigen and antisera obtained from the National Veterinary Service Laboratories (APHIS, USDA, Ames, IA). Serum samples were first treated with receptor destroying enzyme (RDE (II) Denka Seiken, Tokyo, Japan) at a 1:3 dilution followed by incubation for 18 hours at 36ºC and then 56ºC for 1 hour. The HI assay was performed as described (Pedersen, 2008) using 4 HA units/25ul of antigen and a titer >8 was considered

positive (Curran et al., 2014). The VN assays was performed as described (Ramey et al., 2014) using A/mallard/MN/AI11-3933/2011(H5N1) virus. We tested 46 serum samples with the VN assay.

Statistical analyses were done using R v3.0. Agreement between the two bELISA protocols was evaluated using kappa statistics (κ) and percent agreement. Interpretation of the kappa value was based on criteria from Landis and Koch (1977). We used McNemar's $\chi 2$ test to determine if there was a significant difference between the numbers of positive samples detected between the assays.

We detected NP antibodies in 66% (225/340) of serum samples using the NP bELISA (Table 1). In comparison of the two H5-specific bELISA protocols, we detected antibodies in 33% (60/182) of birds using the manufacturer's protocol and in 69% (126/182) using the modified protocol. There was 62% agreement between the two protocols with moderate agreement (κ=0.3 (0.1-0.5); the modified protocol detected significantly more positive samples (McNemar's $\chi 2$=63 $p<0.001$). The H5 antibody prevalence for all 340 samples using the modified bELISA protocol and the HI assay are in Table 1. Because of the low prevalence with the HI assay, we tested a subset (n=46) of samples with the VN assay. The H5 antibody prevalence for this subset was 65% (30/46), 65% (30/46), and 50% (23/46) using the VN assay, modified bELISA protocol, and HI assay, respectively. However, sample selection was based on previous HI results (23 negatives and 23 positives) and are not an accurate representation of the sample population.

Overall, we detected a high IAV NP antibody prevalence in mute swans which supports data by Pederson et al., (2014) who reported a prevalence of 45%. This high antibody prevalence is likely related to the persistence of antibodies, which may be over one year in the absence of

virus circulation (Fereidouni et al., 2010), and age of the sampled birds as mute swans are a long lived waterfowl species (Reese, 1980). We also detected a high prevalence of H5-specific antibodies which was unexpected because H5 IAVs are not frequently reported from waterfowl in North America (Hinshaw et al., 1985).

A previous study detected in experimentally infected ducks detected an increased sensitivity with the H5-specific bELISA using the modified bELISA protocol (Lebarbencon et al. 2013). The data from our study also suggests that the modified protocol for the bELISA for detection of H5-specific antibodies in mute swans and possibly other birds. The low prevalence detected with the HI assay was surprising because a previous study in France detected H5-specific antibodies in >45% of mute swans using the HI assay (Niqueux et al., 2010). The HI assay has performed poorly in experimentally infected mute swans (Kalthoff et al., 2008) and was unable to detect antibodies in naturally exposed sentinel ducks (Globig et al., 2013). Although VN assay, data were similar to those from the modified bELISA protocol and both detected a higher prevalence of antibodies compared with the HI assay. Similar results for comparison of HI and VN assays have been reported in detecting H5 antibodies in humans (Rowe et al., 1999). In addition, the HI assay is likely not the most sensitive assay to detect subtype specific antibodies in mute swans and if used, confirmatory assays should be used.

Overall, our results suggest that mute swans in North American have a high NP antibody prevalence and also a high H5 antibody prevalence. Currently, the significance of this high antibody prevalence is not known but if this reflects a high existing population immunity to H5 viruses, the probability of disease or successful introduction of a highly pathogenic H5N1 viruses to North America may be reduced. Alternatively, these antibodies may protect mute swans from

disease associated with highly pathogenic H5N1 viruses and allow them to circulate without mortality.

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Table 5.1. Prevalence of influenza A virus antibodies to the nucleoprotein (NP) and to the H5-subtype in mute swans (Cygnus olor) from four US states three serological assays.

State	NP bELISA Positive/*n* (%)	Modified bELISA protocol Positive/*n* (%)	H5 Hemagglutination Inhibition Assay Positive/*n* (%)
Michigan	145/182 (80)	136/182 (75)	28/182 (15)
New Jersey	12/68 (18)	27/40 (40)	10/68 (15)
New York	11/12 (92)	9/12 (75)	3/12 (25)
Rhode Island	57/78 (73)	42/78 (54)	23/78 (29)
Total	225/340 (66)	214/340 (63)	64/340 (19)

CHAPTER 6

WOOD DUCKS (*Aix sponsa*) AS POTENTIAL RESERVOIRS FOR INFLUENZA A AND AVIAN PARAMYXOVIRUSES

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Abstract: Influenza A viruses (IAVs) and avian paramyxoviruses (APMVs) are important pathogens of poultry worldwide and both commonly occur in wild waterfowl, especially ducks in the family Anatidae. Although wood ducks (*Aix sponsa*) are members of the Anatidae, their behavior differs from most other species in this family. These differences potentially affect transmission of IAVs and APMVs. Although wood ducks have been previously tested for these viruses, most studies have been restricted to small geographic areas with small sample sizes. There are few reports of serologic testing of this species for antibodies to IAVs and subtype specific antibody prevalence estimates are not currently available for APMVs. We collected cloacal and oropharyngeal swab and blood samples from >700 wood ducks across nine states in the eastern United States. No IAVs were isolated and based on blocking ELISA results, antibodies to IAVs were only detected in 0.2% of samples. In contrast, 23 (3%) APMVs were isolated (22 Newcastle disease virus and one APMV-6) and antibodies to multiple serotypes of APMVs were detected in >60% of samples. After-hatch-year birds had increased odds of testing antibody positive to APMV-4 and APMV-6 compared with hatch-year birds. Female birds had increased odds of testing antibody positive to APMV-4 compared to male birds. Our results indicate that wood ducks likely are not an important host for IAV but are frequently infected with APMVs.

Keywords: avian paramyxoviruses, influenza A viruses, Newcastle disease virus, serology, wood ducks, virus isolation

Introduction

Influenza A viruses (IAVs) and avian paramyxoviruses (APMVs) are important pathogens of poultry worldwide. Wild birds, in the family Anatidae, are recognized reservoirs for IAVs and some serotypes of APMVs (Deibel *et al*., 1983; Hinshaw *et al*., 1985; Olsen *et al*., 2006). However, the contribution of individual waterfowl species in the maintenance of IAVs and APMVs in wild bird populations is not well understood.

Although wood ducks are members of the family Anatidae, their behavioral differs from most other ducks in this family. In the eastern United States (USA) wood ducks breed from Newfoundland, Canada to Florida, and they utilize forested habitats for cavity nesting, roosting, and brood rearing (Bellrose & Holm, 1994). They also have a different feeding ecology, primarily feeding on acorns at certain times of year (Gilmer *et al*., 1978; Delnicki & Reinecke, 1986; Bellrose & Holm, 1994; Granfors & Flake, 1999; Folk & Hepp, 2003). The differences in behavior and habitat use likely affects the epidemiology of both influenza A viruses (IAVs) and avian paramyxoviruses (APMVs) in this species.

Several studies have reported negative results for IAV in wood ducks, but most of these studies included <60 individuals (Rosenberger *et al*., 1974; Bahl *et al*., 1975; Webster *et al*., 1976; Kocan *et al*., 1979; Nettles *et al*., 1985; Hinshaw *et al*., 1986; Slemons *et al*., 1991). Three studies have attempted to isolate virus from >100 wood ducks and two of these studies reported a low prevalence (<2.5%) of viral shedding (Deibel *et al*., 1983; Goekjian *et al*., 2011; Wilcox *et al*., 2011). These studies suggest that wood ducks do not contribute to the maintenance of IAVs in wild bird populations; however, results from three other studies contrast these studies. A study from Canada detected IAVs in 68% (71/104) of wood ducks using RT-PCR (Parmley *et al*., 2008). Two additional studies reported virus isolations from 11.9% of wood ducks sampled

during a one week period on the eastern shore of Maryland and 8% of wood ducks sampled in Alberta, Canada; however the total number of wood ducks sampled was not given (Hinshaw *et al*., 1985; Slemons *et al*., 2003). Finally, most of these studies only tested cloacal swabs; it has since been reported that wood ducks predominantly shed IAVs via the oropharyngeal route (Costa *et al*., 2011).

Two studies have reported IAV antibody test results for wood ducks and in both cases all birds tested negative (Kocan *et al*., 1979; Brown *et al*., 2010). One of these studies only tested serum from three wood ducks by a bELISA (Brown *et al*., 2010) and the other study did not report samples size, but sera were tested by the agar gel immunodiffusion assay (Bahl *et al*., 1975). The agar gel immunodiffusion assay has a low reported sensitivity in waterfowl species (Brown *et al*., 2009, 2010). Experimentally infected wood ducks do develop a detectable antibody response to IAV (Brown *et al*., 2009; Costa *et al*., 2011).

Eight APMV subtypes: Newcastle Disease Virus (NDV; APMV-1), APMV-2, -3, -4, -6, -7, -8, and -9, have been isolated from wild waterfowl; however, NDV, APMV-4, -6, and -8 have been most commonly reported (Alexander *et al*., 1983; Hinshaw *et al*., 1985; Stallknecht *et al*., 1991; Stanislawek *et al*., 2002; Goekjian *et al*., 2011). What little information is known about IAVs and APMVs in wood ducks indicates that APMVs are isolated more frequently than IAVs (Deibel *et al*., 1983; Goekjian *et al*., 2011). Antibodies to APMV in ducks have been reported in four studies (Kocan *et al*., 1979; Vickers & Hanson, 1982; Maldonado *et al*., 1995; Stanislawek *et al*., 2002). Two of these studies were done in North America, but they did not sample wood ducks and only tested for antibodies to NDV (Kocan *et al*., 1979; Vickers & Hanson, 1982).

Currently there are limited and sometimes conflicting results on the prevalence of IAV in wood ducks and a paucity of information on APMV prevalence and subtype diversity in this

species. The objective of this study was to sample wood ducks in the eastern United States and use virus isolation and serology to detect viral shedding and exposure to IAVs and APMVs.

Materials and Methods

Sample collection. Cloacal and oropharyngeal swabs (*n*=724) and whole blood samples (*n*=727) were collected from wood ducks captured by rocket nets in eight states (Florida, Kentucky, Maryland, North Carolina, Pennsylvania, South Carolina, Vermont, and Virginia) between 16 July and 19 September 2009 and by hand during nest box surveys in Maine. Sample collections took place during bird banding programs conducted by the United States Fish and Wildlife Service (USFWS) and several state agencies (see Acknowledgements). Cloacal and oropharyngeal swab samples were placed in the same tube with brain heart infusion broth (BD Biosciences, Franklin Lanes, New Jersey, USA) supplemented with penicillin G (1,000 units/ml), streptomycin (1mg/ml), gentamycin (50mg/ml), kanamycin (50 mg/ml), and amphotericin B (0.025 mg/ml) and were either kept on ice for < 1 day or refrigerated (4 C) for no more than two days before overnight shipping; also, some samples were stored in liquid nitrogen in the field until transported to the Southeastern Cooperative Wildlife Disease Study (University of Georgia, Athens, Georgia, USA). Swab samples were stored at -80˚C until testing. Blood samples were collected from the brachial vein, placed in Vacutainer® serum separator tubes (BD Biosciences), allowed to clot, and stored on ice until serum collection; serum was stored at -20˚C until serological testing.

Virus isolation and identification. Virus isolations were attempted in specific pathogen free embryonated chicken eggs as previously described (Alexander & Senne, 2008). All samples that agglutinated chicken red blood cells were further tested by RT-PCR for IAV. Viral RNA was

extracted from allantoic fluid using QIAmp® Viral RNA Mini Kit (QIAGEN Inc., Valencia, California) and tested for presence of the IAV matrix gene by RT-PCR (Spackman *et al*., 2002). In addition, all hemagglutinating allantoic fluid samples were screened for seven APMV subtypes (NDV, APMV-2, 4, 6, 7, 8, and 9) using a hemagglutination inhibition (HI) assay (Alexander & Senne, 2008). Reference antisera for these APMV subtypes were obtained from the Poultry Diagnostic Research Center (PDRC; University of Georgia, Athens, GA) for NDV and the National Veterinary Service Laboratories (NVSL, Ames, IA). All samples testing positive by APMV HIs were then screened by RT-PCR with serotype-specific primers to the corresponding virus to verify HI results. For NDV RT-PCR for the fusion cleavage site was done as described (Aldous *et al*., 2003) and primers were designed for APMV-6 to amplify a region of the large polymerase gene: APMV6-11510 (5'-AAGGGCTATGCCAAAAAATGTG-3') and APMV6-12014 (5'-GGAAAAATCAATGTCAAAAAGAA-3'). Conditions for the APMV-6 RT-PCR reaction was: 20 min at 42C then 2 min at 94C followed by 94C for 1 min, 1 min at 55C and 1 min at 72C 45 times followed by 72C for 10 min. The NDV fusion cleavage sites of four random isolates were bi-directionally sequenced (Georgia Genomics Facility, University of Georgia, Athens, GA). In addition, the large polymerase gene amplicon from one APMV-6 sample was sequenced to confirm identification.

Serology. Influenza A virus serology was conducted using a commercial bELISA (IDEXX Laboratories, Westbrook, Maine) following the manufacturer's instructions (Brown *et al*., 2009). Serology for the APMVs was conducted using a HI assay against the four APMV subtypes (NDV, APMV-4, -6, and -8) most commonly isolated from wild ducks as described (Alexander and Senne, 2008). Briefly, viruses were cultured in 9-day-old embryonated chicken eggs, amnioallantoic fluids were inactivated with a final concentration of 0.1% β-propiolacton. The pH

of the antigens were adjusted to 7 with 10% sodium bicarbonate solution. Viruses used for antigen preparation were MADU/US(MN)/AI07-4044 for NDV, MADU/US(MN)/AI07-3119 for APMV-4, MADU/US(MN)/AI07-4783 for APMV-6, and APMV-8 antigen was obtained from PDRC. The HI assay was done as previously described, using 8 hemagglutination units of virus/25µl/well (Alexander & Senne, 2008; Warke *et al*., 2008). A sample was considered antibody positive if the titer was $\geq$32. In addition, 663 wood duck serum samples were tested for non-specific agglutination of chicken red blood cells (CRBCs). Positive control reference antisera were obtained from the PDRC for NDV and the NVSL for APMV-4, APMV-6, and APMV-8.

Statistical analysis. All statistical analyses were conducted using R v3.0.2. We used population-averaged generalized estimating equations (GEE) logistic regression to compare differences in antibody prevalence estimates across all four APMV subtypes by age and sex using the Zelig v4.2 package in R (Lam, 2014). Significance was based on the 95% confidence intervals of the odds ratio. We used population-averaged GEE to account for clustering of samples at locations and dates (Hanley *et al*., 2003). Age and gender data were not available for all samples and those samples were not included in these analyses. Interaction between age and sex was examined for all models and significance was analyzed using a Wald test; non-significant interactions were removed from the model.

Results

Virus isolation. We detected hemagglutinating agents in 3% (23/724) of the wood duck swab samples. Amnio-allantoic fluid harvested from these positive samples tested negative for IAV by matrix gene RT-PCR; using the HI assay, 22 of the samples were identified as NDV and one

sample as APMV-6 (Table 1). The HI results were confirmed by RT-PCR for all 23 isolates. Sequenced analysis of the fusion cleavage sites of four NDV isolates (two from NC, and one each from PA, and VT) did not contain additional basic amino acids and were consistent with avirulent NDV (110-GGERQERL-117). The large polymerase gene segment amplified from the one APMV-6 isolate was 91% similar to APMV-6/duck/HongKong/18/199/77 (GenBank # EU622637). All isolates were from hatch-year birds; age data were not available for seven of the NDV positive birds.

Serology. We detected antibodies to the IAV nucleoprotein in 0.3% (2/727) of sampled wood ducks (Table 1). In contrast, antibody prevalence was >66% for NDV, APMV-4, -6, and -8, except for APMV-4 in Maine, Vermont, and Virginia and APMV-8 in Vermont and Virginia (Figure 1). The distribution of antibody titers to these viruses is shown on (Figure 2). We did not detect any interaction between age and gender in our population averaged GEE logistic regression model (Wald test $p>0.05$); however, we did detect differences in antibody prevalence associated with age and gender. After-hatch-year birds had increased odds of exposure to APMV-4 and APMV-6 compared to hatch-year birds and females had increased odds of exposure to APMV-4 compared to males (Table 2). Although not significant, after-hatch-year birds had increased odds of exposure to NDV compared to hatch-year birds (Table 2). We did not detected any non-specific agglutination of CRBCs from the 663 wood duck serum samples tested, indicating that the risk of a false positive reaction due to non-specific agglutination was unlikely.

Discussion

The difference in viral shedding and exposure between IAVs and APMVs we detected was interesting. Both of these viruses use waterfowl as hosts and are believe to be fecal-orally transmitted (Webster *et al.*, 1978; Dai *et al.*, 2013). Water and sediment contaminated with virus is believed to be the main route of transmission for both of these viruses in waterfowl. Persistence of these viruses in the environment likely plays a large role in transmission of these viruses. In experimentally controlled setting NDV has been shown to persist longer in soil sediment and feces than IAVs (Nazir *et al.*, 2011). This has not been thoroughly evaluated with all APMVs serotypes, but this increased environmental persistence is likely responsible for the perceived increase in circulation of APMVs in wood ducks.

Consistent with previous studies (Kocan *et al*., 1979; Deibel *et al*., 1983; Slemons *et al*., 1991; Goekjian *et al*., 2011; Wilcox *et al*., 2011), we detected limited evidence of IAV circulation among wood duck populations sampled. These results are supported by the very low antibody prevalence we observed across a very broad geographical area which included northern latitudes where IAVs are more likely to be detected in waterfowl (Deibel *et al*., 1983; Hinshaw *et al*., 1985; Wilcox *et al*., 2011). As a comparison, antibody prevalence in mallards (*Anas platyrhynchos*) and Canada geese (*Branta canadensis*) sampled during these same time periods is can exceed 50% and 20%, respectively (Brown *et al*., 2010; Kistler *et al*., 2012). Although, the duration of the detectable IAV antibody response is not well defined for wood ducks and may partially explain the low prevalence, antibodies to IAV nucleoprotein have been detected for >1 year in naturally exposed mallards (Fereidouni *et al*., 2010) and for 21 days in experimentally infected wood ducks and mallards (Costa *et al*., 2011). These results suggests that wood ducks are likely spillover hosts of IAVs and it is possible that previous reported isolations of IAV from

this species reflect such spillover (Deibel *et al*., 1983; Hinshaw *et al*., 1985; Wilcox *et al*., 2011). Two of the three studies that isolated IAV from wood ducks were done in northern latitudes, during peak IAV shedding in waterfowl (late summer early fall), and in aquatic habitats with large numbers of mallards, a known IAV reservoir species (Deibel *et al*., 1983; Hinshaw *et al*., 1985; Wilcox *et al*., 2011). Another study that detected a high prevalence of IAV (68%) using RT-PCR also was conducted under similar conditions across Canada (Parmley *et al*., 2008). Additionally, these studies collected many of their samples from baited ducks, which could increase transmission of IAV among waterfowl species (Soos *et al*., 2012).

Waterfowl are considered hosts for at least four APMVs subtypes (Alexander *et al*., 1983; Hinshaw *et al*., 1985; Stanislawek *et al*., 2002), but only NDV and APMV-4 have been reported from wood ducks (Goekjian *et al*., 2011). We isolated NDV and APMV-6 during this study but our serological results provide evidence that wood ducks are commonly infected with other APMV serotypes. Our APMV virus isolation prevalence was lower than two previous studies that sampled wood ducks during the same time of year. One study conducted in North Carolina detected viral shedding in $\geq$9% of wood ducks and the second study conducted in New York isolate APMVs from >8% of wood ducks (Deibel *et al*., 1983; Goekjian *et al*., 2011). In comparison to our study, wood ducks in these two studies were sample from the same areas for an extended period of time (> 1 month) while we only sampled for $\leq$2 days at each location. Our short sampling period makes it possible that we missed the peak of virus circulation. In wood ducks, seasonal peaks in NDV prevalence (<15% to >40%) that subsided rapidly were reported from birds sampled in North Carolina; however, a high prevalence could not be detected 7-10 days after these detected peaks (Goekjian *et al*., 2011).

Most of the NDV viruses we isolated came from hatch-year birds, which is consistent with other reports of APMVs in waterfowl (Deibel *et al*., 1983; Stallknecht *et al*., 1991). Virulent NDV is an important pathogen in poultry, which is characterized by the addition of basic amino acids at the fusion protein cleavage site (Collins *et al*., 1993). In waterfowl NDV isolates usually have fusion protein cleavage sites consistent with avirulent NDV (Jindal *et al*., 2009). Our fusion cleavage site analysis supports this. In North American wild birds, virulent NDV is only associated with double-crested cormorants (*Phalacrocorax auritus*) (Kuiken et al., 1998; Glaser *et al*., 1999; Allison *et al*., 2005). Avian paramyxovirus-6 has been commonly reported from various waterfowl species (Goekjian *et al*., 2011; Hinshaw *et al*., 1985), but has not been previously reported a wood duck.

We detected a high prevalence antibodies to four APMV serotypes in this study. These data suggest that wood ducks are frequently exposed to four serotypes of APMVs. The lower level of antibody detection (<50%) in some states to APMV-4 and APMV-8 cannot be explained but these prevalence estimates are based on only one or two sampling locations per state and differences may reflect local spatial variation. In Canada geese, significant variation of IAV antibody prevalence has been reported at sample locations within 10km of each (Kistler *et al*., 2012). Only low prevalence estimates (<16%) of NDV antibodies in North American waterfowl have previously been reported (Kocan *et al*., 1979; Vickers & Hanson, 1982), and to our knowledge, there has been no reports of testing for antibodies to other APMVs in North American waterfowl. However, in New Zealand antibody prevalences >50% to NDV, APMV-4, APMV-6, and APMV-8 were reported in mallards (Stanislawek *et al*., 2002) and in Spain antibody prevalences >30% to APMV-6 and APMV-8 were reported in waterfowl species (Maldonado *et al*., 1995). Cross reactivity has been reported in APMVs (Lipkind & Shihmanter,

1986), and the high antibody prevalence we detected might reflect some degree of cross-reaction among APMV serotypes.

The antibody prevalence to all APMVs was high in hatch-year birds, indicating that infection likely occurred during the summer. Transmission of NDV in wood duck populations during the summer has been previously reported (Goekjian *et al*., 2011). We did detect differences in antibody prevalence by age and gender for APMV-4 by age for APMV-6; however, these results should be interpreted with caution. The lower confidence limits for the estimated odds ratios are very close to one. In addition, we did not confirm infection of wood ducks with either APMV-4 or APMV-8 through virus isolation. Although our serological data supports wood ducks playing a significant role in the epidemiology of multiple serotypes of APMVs, there is insufficient historic virus isolation data to support this. Part of this relates to the fact that most APMV isolates are a byproduct of IAV targeted surveillance and research. These studies have most often taken place in late summer and fall and this seasonality may not correspond to times of peak APMV shedding (Hinshaw *et al*., 1985; Wilcox *et al*., 2011). This situation will not be improved by the use of molecular screening techniques for IAV detection that will for the most part eliminate the possibility of APMV detection.

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Table 6.1. Results from virus isolations for influenza A viruses (IAVs) and avian paramyxoviruses (APMVs) in wood ducks and antibody prevalence as determined by a blocking ELISA.

	Virus Isolation		Serology
State	IAV (%)	APMV (%)	IAV (%)
Florida	0/92 (0%)	0/92 (0)	0/86 (0)
Kentucky	0/79 (0%)	1/79 (1.3)	0/77 (0)
Maine	0/29 (0%)	0/29 (0)	0/29 (0)
Maryland	0/75 (0%)	6/75 (6.7)	0/75 (0)
North Carolina	0/84 (%)	7/84 (8.3)	0/88 (0)
Pennsylvania	0/28 (0%)	1/28 (3.6)	0/27 (0)
South Carolina	0/161 (0%)	1/161 (0.6)	1/169 (0.6)
Vermont	0/110 (0%)	7/110 (6.4)[a]	1/110 (0.9)
Virginia	0/66 (0%)	0/66 (0)	0/66 (0)
Total	0/724 (0)	23/724 (3)	2/727 (0.3)

[a]One isolate APMV-6 the other isolates are Newcastle disease virus

Table 6.2. Results from logistic regression utilizing generalized estimating equations for antibodies to avian paramyxovirus (APMV) in wood ducks comparing after-hatch-year (AHY) to hatch-year (HY) and female to male.

Virus	Age	Odds ratio (95%CI) Positive/*N* (%)	Gender	Odds ratio (95%CI) Positive/*N* (%)
Newcastle Disease Virus	AHY	1.5 (1.0, 2.1) 250/284 (88)	Female	0.6 (0.3, 1.1) 176/208 (85)
	HY	Referent[a] 259/294 (88)	Male	Referent 333/370 (90)
APMV-4	AHY	1.5 (1.1, 2.0) 195/282 (69)	Female	1.4 (1.1, 1.9) 141/204 (69)
	HY	Referent 158/288 (55)	Male	Referent 212/366 (58)
APMV-6	AHY	2.3 (1.1, 4.8) 273/277 (99)	Female	0.7 (0.4, 1.3) 190/197 (96)
	HY	Referent 265/276 (96)	Male	Referent 348/356 (98)

APMV-8	AHY	1.2 (0.9, 1.6)	Female	1.2 (0.9, 1.4)
		194/264 (73)		139/191 (73)
	HY	Referent	Male	Referent
		163/270 (60)		218/343 (64)

[a] Referent is the comparison group

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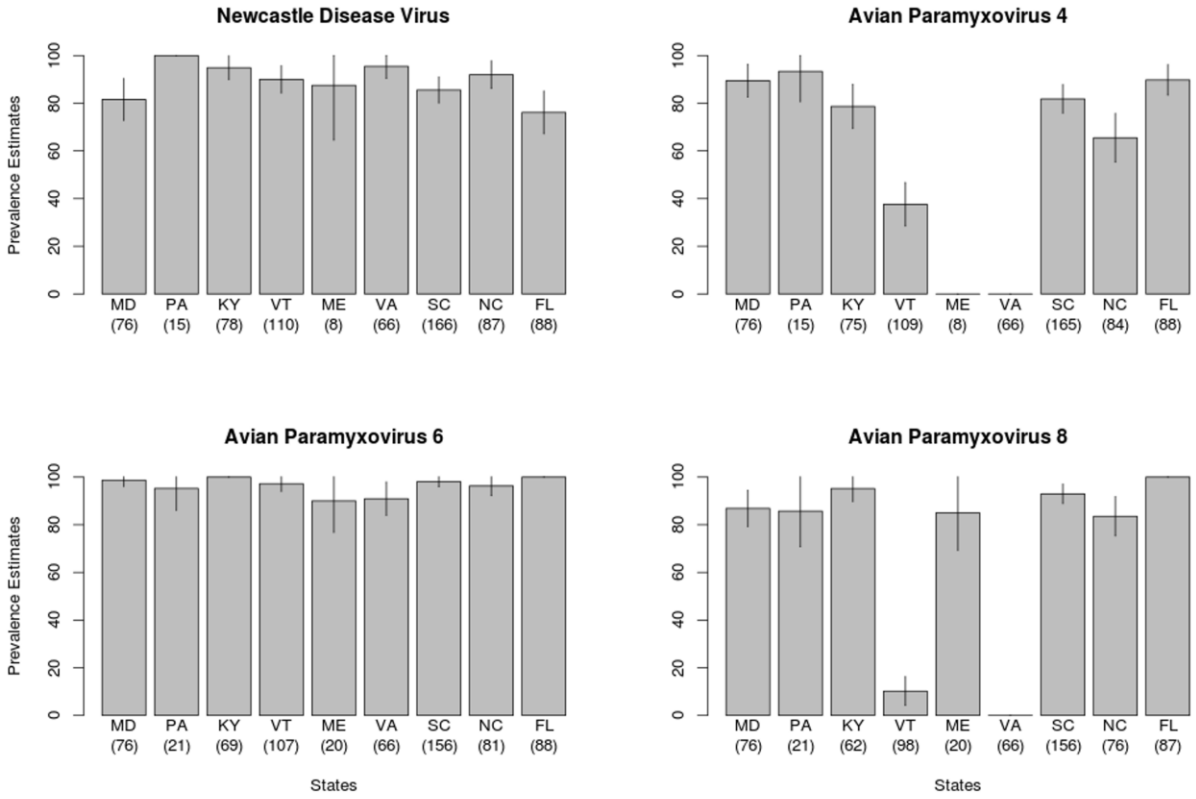

Figure 6.1. Prevalence estimates from avian paramyxoviruses by state as determined by hemagglutination inhibition assay. Error bars are based on 95% confidence intervals using the standard error and were capped at 100%. Numbers in parenthesis are number of birds sampled

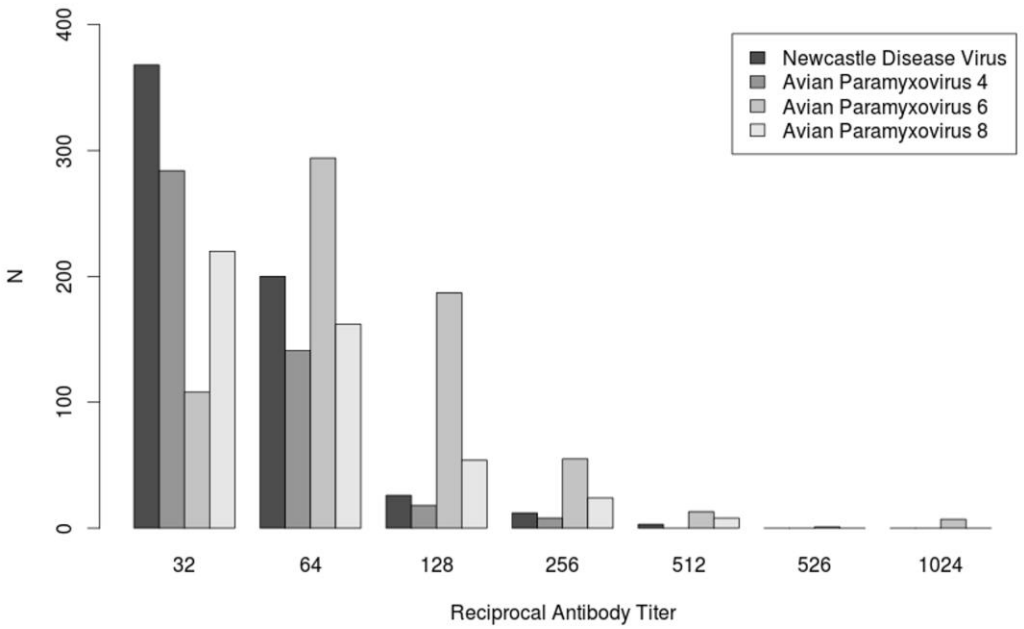

Figure 6.2. Reciprocal antibody titers of wood ducks to avian paramyxoviruses as determined using hemagglutination inhibition assay to four serotypes.

CHAPTER 7

CONCLUSIONS

The major objective of this research was to use serological assays to better understand the epidemiology of influenza A viruses (IAVs) and avian paramyxoviruses (APMVs) in wild waterfowl. Both IAVs and APMVs are known to use wild waterfowl as hosts and are important pathogens of poultry.

Study 1 (Chapter 3)

The main objective of this study was to further evaluate annual variation and spatial variation of IAV nucleoprotein antibodies in Canada geese within Pennsylvania. A similar previous study found no significant variation in antibody prevalence between years and that geese sampled in urban areas had higher odds of exposure than geese sampled from rural areas. The results from this study also support low variation among years on the statewide scale and at locations sampled multiple years. The only environmental variable found to significantly influence antibody prevalence in Canada geese was % developed land.

The lack of significant variation in antibody prevalence seen annually is likely due to the persistence of antibodies and that Canada geese are long lived species. These same conclusion are likely the reason for increased odds of exposure in Canada geese from urban areas. Overall, the lack of variation in antibody prevalence and increased exposure in developed areas means that serology is not sensitive enough to detect variations in transmission either annually or on a small spatial scale.

Study 2 (Chapter 4)

The main objective of this study was to evaluate subtype-specific antibody prevalence in Canada geese to determine which subtypes geese are frequently exposed and if those subtypes reflect the diversity reported from dabbling ducks. The data obtained for this study demonstrated: 1) Canada geese are frequently exposed to H5 IAVs, 2) Canada geese are frequently exposed to the three most commonly isolated IAV subtypes found circulating in dabbling ducks (H3, H4, and H6) and are not commonly exposed to other subtypes, which are isolated at low frequencies from dabbling ducks, 3) H5 IAV antibodies in Canada geese are wide spread and commonly detected, likely indicating H5 IAVs circulate more frequently than IAV detection in dabbling indicates. These findings support and expand on previous research showing that IAVs antibodies in Canada geese overlap with recorded virus isolation in dabbling ducks on a spatial scale and by subtypes (Kistler et al., 2012). However, the H5 IAV antibody results were interesting, because this subtype is not frequently detecting during surveillance studies and may indicate a different circulation pattern for this subtype. Together, these results suggest that Canada geese share a common IAV exposure with dabbling ducks and geese may be used to detect IAV circulation on a regional scale.

Study 3 (Chapter 5)

This study was the first large-scale surveillance study for H5-specific antibodies in North American mute swans. Mute swans were found to have a high prevalence of antibodies to the nucleoprotein and using two different assays were found to have a high prevalence of H5-specific antibodies. A previous study by Pedersen et al., (2014) also found a high NP antibody prevalence in mute swans from North American. This is likely related to antibody persistence

and mute swans being a long lived species. The high H5-specific antibody prevalence was surprising because these viruses are not frequently reported from surveillance studies aimed at detecting IAVs. This high prevalence may play a role in preventing the introduction of highly pathogenic H5N1 in North American or reduce the impact it may have on wild bird population if it were to ever make it to North America.

Study 4 (Chapter 6)

The main objective of this study was to evaluate the role of wood ducks in the epidemiology of IAV and APMVs using serological assays and virus isolation. The results showed that wood ducks are not frequently exposed to AIVs, but are frequently exposed four APMV serotypes (Newcastle Disease Virus, APMV-4, APMV-6, and APMV-8). These data, in conjunction with previous research (Deibel et al., 1983), suggest exposure of wood ducks to IAVs is likely dependent on location and presence of IAV shedding in other dabbling ducks. Additionally, wood ducks likely play a large role in the epidemiology of APMVs; however, virus isolation data is lacking.

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