POLYMORPHISM IN THE DUCK MX GENE AND ASSOCIATION WITH INFLUENZA INFECTION

By

Danielle M. Dillon

RECOMMENDED:

Advisory Committee Chair

Chair, Department of Biology and Wildlife

APPROVED:

Dean, College of Natural Science and Mathematics

Dean of the Graduate School

April 2, 2010

Date

POLYMORPHISM IN THE DUCK MX GENE AND ASSOCIATION WITH INFLUENZA INFECTION

A

THESIS

Presented to the Faculty

of the University of Alaska Fairbanks

in Partial Fulfillment of the Requirements

for the Degree of

MASTER OF SCIENCE

By

Danielle M. Dillon, B.S.

Fairbanks, AK

May 2010

ABSTRACT

Myxovirus-resistant (Mx) proteins are induced by interferon and inhibit viral replication as part of the innate immune response to viral infection in many vertebrates. Influenza A virus appears to be especially susceptible to Mx antiviral effects. We characterized exon 13 and the 3' UTR of the Mx gene in wild ducks, the natural reservoir of influenza virus and explored its potential relevance to influenza infection. We observed a wide range of intra- and interspecies variation. Total nucleotide diversity per site (π/bp) was 0.0014, 0.0027, 0.0044, 0.0051, and 0.0061 in mallards, northern shovelers, northern pintails, American wigeon, and American green-winged teals, respectively. There were 61 haplotypes present across all five species and four were shared among species. Additionally, we observed an association between Mx haplotype and influenza infection status in northern shovelers. However, we found no evidence of balancing or diversifying selection in this region of the Mx gene. Characterization of the duck Mx gene is an important step in understanding how the gene may affect disease resistance or susceptibility in wild populations. Furthermore, given that waterfowl act as a natural reservoir for influenza virus, the Mx gene could be an important determinant in the ecology of the virus.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

ACKNOWLEDGEMENTS

This thesis is the result of hard work and dedication of many people. First, I would like to thank my advisory committee chair, Jonathan Runstadler, for providing invaluable guidance and support. He ignited my interest in scientific research as a young undergraduate student and gave me the opportunity to further pursue that interest as a graduate student in his lab. I am extremely grateful to the members of my committee, Karsten Hueffer and Diana Wolf, for helping to make me a competent scientist. They asked the questions that needed to be asked and pushed me when I needed to be pushed. I consider myself very lucky to have a committee that worked so hard to help me succeed.

The collection and analysis of cloacal samples was an enormous undertaking and would not have been possible without the involvement of field crews at Yukon Flats National Wildlife Refuge and Minto Flats State Game Refuge and members of the Happ Laboratory at University of Alaska Fairbanks. I would like to thank Mike Petrula for his continued support and cooperation in lending his Minto Flats expertise. I am grateful to Nancy Gundlach, Ana Marx, Leif Vick, and Paige Gingrich for preparing, processing and analyzing thousands of cloacal swabs. Trista Welsh and Brandt Meixell collected a large collection of feathers and blood samples that were integral to this project.

I owe a tremendous thanks to friends and lab mates who helped me in more ways than they can imagine, especially: Keiko Herrick, Lori Gildehaus, Mark Nelson, Paige Gingrich, Florian Aldehoff, Leif Vick, Carrie Topp, Alison Tribenbach, Sarah Nash, and Pat and Emma Spencer.

Finally, I would like to thank my husband, Matthew Dillon, and my parents, Laurie and Keith Mondloch, for their incredible love and support. Matt is my biggest cheerleader and I am honored to be sharing life's adventures with such a wonderful man. My parents kept life in perspective for me over these last three years and supported my choices unconditionally. They truly helped make this project come together successfully.

INTRODUCTION

Innate immunity and the Mx gene

The innate immune system is an organism's first line of defense against viral and bacterial pathogens. Physical and chemical barriers are the first hurdles a pathogen must overcome. Once inside the organism, pathogen-associated molecular patterns (PAMPs) are recognized by host pattern recognition receptors (PRRs). PAMPs make it possible for the innate immune system to recognize entire classes of pathogens because they generally do not vary and are usually vital in some way to the pathogen for infectivity or structural integrity (e.g. membrane components, nucleic acids, flagella). When a PAMP is recognized by a PRR, a signaling cascade begins that promotes differential gene expression, results in communication with the adaptive immune system, and ultimately, triggers an immune response to control infection by the pathogen (Mogensen, 2009).

Upon viral infection, the innate immune system recognizes characteristic viral PAMPs such as double-stranded RNA, single-stranded RNA, DNA, and surface glycoproteins (Mogensen, 2009). Recognition results in the production of proinflammatory cytokines and interferon (IFN). Type I (α/β) IFN is a potent activator of antiviral proteins and other immunomodulatory cytokines, and its production is critical to mounting an effective response to viral infection (reviewed by Haller et al., 2006). The most extensively studied proteins induced by IFN are protein kinase R (PKR), 2'-5'-oligoadenylate synthetase (2-5 OAS), and Mx GTPase (Haller et al., 2007a).

Mx GTPases have proven to be key mediators in the antiviral innate immune response (Lindenmann, 1962; Lindenmann, 1964). After an inbred mouse strain proved to be resistant to infection with influenza A virus, an infection lethal to other laboratory mice, a gene called *Mx1* (myxovirus-resistant) was found to be responsible for the resistance. Later work revealed the mouse genome contained a second Mx gene, *Mx2*, and both genes are present and intact in wild mice (Jin et al., 1998; Jin et al., 1999). Subsequently, the presence of Mx was confirmed in most vertebrate species, including humans, birds and fish (Staeheli et al., 1989; Horisberger, 1992; Bazzigher et al., 1993; Bernasconi et al., 1995).

Most species possess two or more Mx genes, but not all are antivirally active. Humans have one antivirally active form, *MxA*, and a non-antiviral form, *MxB* (Aebi et al., 1989). Rats are unique as they have two antivirally active forms, *Mx1* and *Mx2*, and one non-antiviral form, *Mx3* (Meier et al., 1988). This is in contrast to chickens and ducks, which each only have one Mx gene, and controversy exists over their antiviral activities (Bazzigher et al., 1993; Bernasconi et al., 1995; Ko et al., 2004; Benfield et al., 2008). At this time, it is unknown if the non-antiviral forms of Mx serve other biological functions.

The sequence and structure of vertebrate Mx proteins are generally conserved. They are a subclass of the dynamin superfamily and thus contain dynamin-like features such as an N-terminus GTP-binding domain, a central interactive domain, and a C-terminus effector domain with a leucine zipper motif (Haller et al., 2007c). Also common

to dynamin-like molecules is the ability to self-assemble into oligomers; Mx proteins form helical and ring-like structures in vitro (Kochs, 2002). Forming oligomers appears to be important for long-term stability and hydrolyzing GTP, as mutating the MxA leucine zipper region (L612K) stops self-assembly and GTP hydrolysis; and the protein is rapidly degraded (Janzen et al., 2000; Haller et al., 2007c).

Mx protein subcellular localization differs between species, and in rodents, it determines antiviral specificity. Human MxA is found in the cytoplasm and is effective against a wide range of RNA and DNA viruses (Haller et al., 2007b). Mouse Mx1 is found in the nucleus and inhibits influenza virus and Thogoto virus, while Mx2 localizes to the cytoplasm and inhibits vesicular stomatitis virus (VSV) and Hantaan virus (Pavlovic et al., 1992; Zürcher et al., 1992; Haller et al., 1995; Jin et al., 1999; Jin et al., 2001). Chicken Mx protein localizes to the cytoplasm and early reports stated that it lacked antiviral activity (Bernasconi et al., 1995). More recently, Ko et al. (2004) proposed that antiviral activity against influenza virus and VSV was determined by a nonsynonymous substitution (S631N) in the effector domain. This finding was not supported by a subsequent study (Benfield et al., 2008). However, researchers are interested in the considerable polymorphism at the chicken Mx locus and the impact it might have on protein function in a wide variety of avian species.

Influenza virus and dabbling ducks

Influenza A viruses are a group of RNA viruses that can infect warm blooded animals such as birds, horses, swine, and humans. Due to its segmented genome and

error-prone RNA polymerase, influenza viruses are subject to genetic recombination, mutation, and reassortment (Webster et al., 2007). The genetic changes observed in the virus may depend on the host it is adapted to. Influenza viruses from mammals and domestic poultry show evidence of reassortment, recombination, insertion/deletion, while only mutation and reassortment have been seen in wild birds (Obenauer et al., 2006). Influenza viruses can be divided into host defined lineages: wild aquatic birds, humans, pigs, horses, and domestic poultry, and geographical superfamilies: Eurasian and North American (Webster et al., 2007). These divisions are certainly not static; transmission occurs between hosts and occasionally between geographical clades (reviewed by Webster et al., 2007).

It is hypothesized that there is a cycle of reservoirs, hosts and intermediates that facilitate adaptation and transmission. Avian influenza viruses can be passed from wild waterfowl to chickens and then on to mammals, including humans. Pigs are thought to be an important intermediate host because their lungs contain both α2-6 sialic acid (human-like influenza receptors) and α2-3 sialic acid (avian-like influenza receptors). Chickens also possess both receptors, but α2-6 sialic acid residues are the dominant type in the tracheal epithelium (Kuchipudi et al., 2009). Other means and/or barriers to transpecies transmission are being investigated, but studies of the host factors that impact transmission between wild bird reservoirs of influenza are very limited.

Wild waterfowl are considered the natural reservoirs of influenza virus and harbor all known subtypes (Webster et al., 1992). They appear to be infected by and shed low

pathogenic virus without displaying overt clinical symptoms (Webster et al., 1992). Prevalence of infection is higher in ducks than other birds, and greater in dabbling ducks (genus *Anas*) than diving ducks (Webster et al., 1992; Olsen et al., 2006). This difference in prevalence may be due to ducks' use of aquatic environments, feeding behavior, and migratory patterns (Olsen et al., 2006). Additionally, ducks can be coinfected with different influenza subtypes (Sharp et al., 1997). Frequent co-infection would provide an environment conducive for genetic reassortment (Hatchette et al., 2004).

Given the unique role of ducks in the ecology of the influenza virus, there is surprisingly little work investigating host immunogenetics and the impact it may have on influenza infection and disease. Overall, data are sparse concerning the genetics of wild duck populations. The Mx locus is of interest due to its historical and experimental association with influenza virus and the questions left unanswered by the previous investigation of duck Mx.

Investigations in other species involved in the ecology of influenza are beginning to reveal the complexity of the Mx locus. The porcine Mx1 locus contains alleles with differential antiviral activity against influenza and one isoform displayed a stronger repression of viral replication than human MxA (Palm et al., 2007). Additionally researchers have described extensive polymorphism and evidence of selection on chicken Mx (Ko et al., 2002; Hou et al., 2007; Li et al., 2007; Berlin et al., 2008), which suggests this may be the case at the duck Mx locus as well. Finally, associations are seen between particular chicken Mx alleles and both commercial traits and antibody titers to

vaccinations (Livant et al., 2007; Qu et al., 2009). It is clear a precedent exists to study variation at the Mx locus and associations with influenza virus.

This study had three objectives regarding the Mx gene in wild ducks. The first was to characterize a region of the gene in five wild duck species commonly infected by influenza A viruses by sequencing DNA. The second was to assess variation and evidence of selection in the duck Mx sequences. The final objective was to investigate whether an association exists between Mx allele and influenza infection status. Five dabbling duck species were used: American green-winged teal (*Anas crecca carolinensis*), American wigeon (*Anas americana*), mallard (*Anas platyrhychos*), northern pintail (*Anas acuta*), and northern shoveler (*Anas clypeata*). We sampled at summer breeding grounds in Interior Alaska where large numbers of these birds converge and where dabbling ducks at these sites historically have a high prevalence of influenza infection (Ito et al., 1995; Runstadler et al., 2007).

CHAPTER 1: Mx gene diversity and influenza association among five wild dabbling duck species (*Anas* spp.)[1]

ABSTRACT

Mx (myxovirus-resistant) proteins are induced by interferon and inhibit viral replication as part of the innate immune response to viral infection in many vertebrates. Influenza A virus appears to be especially susceptible to Mx antiviral effects. We characterized exon 13 and the 3' UTR of the Mx gene in wild ducks, the natural reservoir of influenza virus and explored its potential relevance to influenza infection. We observed a wide range of intra- and interspecies variation. Total nucleotide diversity per site (π/bp) was 0.0014, 0.0027, 0.0044, 0.0051, and 0.0061 in mallards, northern shovelers, northern pintails, American wigeon, and American green-winged teals, respectively. There were 61 haplotypes present across all five species and four were shared among species. Additionally, we observed a significant association between Mx haplotype and influenza infection status in northern shovelers. However, we found no evidence of balancing or diversifying selection in this region of the Mx gene. Characterization of the duck Mx gene is an important step in understanding how the gene may affect disease resistance or susceptibility in wild populations. Furthermore, given that waterfowl act as a natural reservoir for influenza virus, the Mx gene could be an important determinant of the viral ecology and evolution.

---

[1] Prepared for submission to *Infection Genetics Evolution* as: Dillon, D., Runstadler, J., Mx gene diversity and influenza association among five wild dabbling duck species (*Anas* spp.)

Keywords: Mx, Duck, *Anas*, Population genetics, Influenza, Nucleotide diversity

BACKGROUND

Mx proteins play a key role in the innate immune response to viral infection. Discovered in 1962 in an inbred mouse strain resistant to influenza infection (Lindenmann, 1962), Mx proteins have since been shown to provide resistance to a wide array of both DNA and RNA-based viruses (reviewed by Haller et al., 2007). They are readily induced by type I and type III interferon (IFN) and where examined, Mx proteins have been found in mammals, birds and fish (reviewed by Haller et al., 2007). Many organisms have both a cytoplasmic and nuclear form of Mx protein. Once induced, Mx proteins aggregate in their respective subcellular localizations. Cytoplasmic Mx associates with the smooth endoplasmic reticulum (Stertz et al., 2006), whereas nuclear Mx localizes to distinct subnuclear compartments (Engelhardt, 2001; Engelhardt, 2004).

Prevention of viral replication is dependent on the co-localization of Mx proteins and the viral replication stage. In the case of orthomyxoviruses and bunyaviruses, a physical interaction occurs between Mx and the viral nucleoprotein (NP) component of the nucleocapsid (Haller et al., 2008). If both occur in the same location, Mx traps viral nucleoproteins and shuttles them elsewhere in the cell (Haller and Kochs, 2002). It is hypothesized that Mx can also bind and cover nuclear localization signals on the viral nucleoprotein, blocking entrance to the nucleus (Kochs and Haller, 1999). The nucleoprotein of most viruses is a generally well-conserved portion of the virus because of its critical role in the virus' structure. However, in a recent study of over 2500

influenza A NP amino acid sequences, it was found that sequence conservation varies considerably by protein domain; 80.7% of residues in the head domain are conserved, whereas only 66.1% and 67.5% of residues in the body and tail loop domains, respectively, are conserved (Ng et al., 2009). It is unknown at this time with which domain Mx interacts, but by targeting the nucleoprotein rather than other viral proteins, it is thought that the Mx protein is better able to maintain viral recognition and prevent viral immune evasion (Sadler and Williams, 2008).

Interaction with viral components is mediated by the effector domain of the Mx protein. The effector domain, also called the leucine zipper region, is coded in exon 13 of the Mx gene. This region is characterized by a series of leucines that appear to be involved in self-assembly and oligomerization. It is thought the formation of oligomers creates a stable pool from which monomeric Mx proteins can be recruited (Janzen et al., 2000; Kochs, 2002). The effector domain also contains amino acids important for viral target recognition. Glutamic acid at position 645 in the human Mx protein (MxA) was mutated to arginine and the protein became antivirally inactive against vesicular stomatitis virus and LaCrosse virus, but still inhibited influenza and Thogoto virus (Zürcher et al., 1992; Kochs et al., 2002).

Antiviral activity of Mx protein varies among organisms and it is unknown at this time why these differences exist. Only the cytoplasmic form (MxA) in humans is antivirally active (Pavlovic et al., 1990), while both forms (nuclear, Mx1; cytoplasmic, Mx2) are active in mice (Arnheiter et al., 1980). Although chicken Mx proteins were not

thought to be antivirally active (Bernasconi et al., 1995), work has shown a single amino acid change (S631N) determines antiviral activity against influenza virus and vesicular stomatitis virus *in vitro* (Ko et al., 2004). However, this finding remains controversial as additional researchers have not successfully replicated the experiment (Benfield et al., 2008). The demonstration that single amino acid changes may determine antiviral activity of some forms of the Mx protein against many different types of virus suggests that genetic variation in the domains of the protein that interact with viral proteins for innate defense may be critical to co-evolving host-pathogen relationships such as what appears to exist with dabbling ducks and influenza.

Duck Mx was also reported as inactive against influenza infection (Bazzigher et al., 1993). However, that study was limited by the extremely small sample size and effectively neglected the possible effects of inter-individual variation in the innate immune response. In addition, to test for resistance to influenza, chicken and turkey-adapted influenza virus strains were used in mouse and chicken-derived cell lines, but it is now well established that the immune response to influenza is strain dependent in both ducks and chickens (reviewed by Cardona et al., 2009). This fact makes it equally likely that the effects observed in a limited number of tissues and a single species were highly dependent on the lab adapted strain of virus used. Bazzigher et al. (1993) discovered alternatively spliced versions of two duck Mx alleles which resulted in four distinct proteins. Despite this finding, no additional individuals or species were tested to further investigate the impact of multiple alleles on resistance to infection. Taken together, the

prior studies suggest the function of the Mx protein in wild duck populations needs to be clarified.

Wild duck populations are an important part of the ecology of the influenza virus. Prevalence of influenza is highest in ducks compared to other birds and in juveniles compared to other age classes (Webster et al., 1992; Olsen et al., 2006). Dabbling ducks (genus *Anas*) are infected with influenza more often than diving ducks (Olsen et al., 2006). This difference in prevalence may be due to dabbling ducks' feeding and migratory behavior (Olsen et al., 2006). Dabbling ducks serve as the natural reservoir for low pathogenic strains and it appears they likely carry and shed the virus without overt symptoms (Webster et al., 1992). When experimentally infected with influenza virus, those duck species tested produced a relatively weak and transitory antibody response (Kida et al., 1980). The primary innate immune response to low pathogenic influenza infection in ducks remains largely unexplored.

Multiple studies suggest the Mx protein is fundamental to the innate immune response to influenza infection in mice, rats, humans, and pigs (Lindenmann and Klein, 1966; Meier et al., 1988; Pavlovic et al., 1990; Nakajima et al., 2007). Given the differing level of conservation in the influenza nucleoprotein and the ability of Mx to recognize the nucleoprotein of many different viruses, it could be advantageous for a population to harbor multiple Mx alleles. A particular Mx allele may be more effective in binding and preventing viral replication than others. This has been described in chicken MHC where certain haplotypes are associated with a resistance to Marek's disease and infectious

bronchitis virus (Bacon et al., 2004; Livant et al., 2004). Thus, polymorphism at the Mx locus in ducks could prove to be related to their ability to effectively control viral infection, as seen with porcine and chicken Mx (Ko et al., 2002; Nakajima et al., 2007; Palm et al., 2007). Characterizing the Mx gene in wild duck species is an important step in investigating genetic susceptibility or resistance to influenza infection at this locus.

In this study, we sequenced and analyzed a portion of the Mx gene which codes for the leucine zipper region of the translated protein which is thought to direct the antiviral effects of the Mx protein in other species. Using those sequences, we were also able to test for an association between Mx allele and influenza infection status. We used samples from multiple individuals with a known infection status representing five dabbling duck species: American green-winged teal (*Anas crecca carolinensis*, hereafter teal), American wigeon (*Anas americana*, hereafter wigeon), mallard (*Anas platyrhynchos*), northern pintail (*Anas acuta,* hereafter pintail) and northern shoveler (*Anas clypeata*, hereafter shoveler). We sampled at summer breeding grounds in Interior Alaska where large numbers of these birds converge and where, historically, dabbling ducks in these sites have a high prevalence of influenza infection (Ito et al., 1995; Runstadler et al., 2007). Nucleotide diversity at the Mx locus would support further investigation of the functional differences between alleles and their impact on influenza infection and evolution in wild duck populations, influenza's major reservoir in nature.

METHODS

Sample collection

We used samples from three existing collections: genomic DNA extracted from blood and feathers collected from ducks at sites in the Yukon Flats National Wildlife Refuge (66°20'N, 147°58'W) in May and June 2006, genomic DNA extracted from blood and feathers collected from live-trapped ducks at sites in the Minto Flats State Game Refuge (64°53′N, 148°46′W) in August 2006, and blood collected from ducks post-mortem at Minto Flats in September 2006 and 2007. Minto Flats and Yukon Flats are both located in interior Alaska and are important breeding areas for more than 20 waterfowl species (Clausen et al., 1992; Petrula, 1994). Table 1 lists samples used in laboratory analysis.

Yukon Flats

Wigeon and shovelers were live-captured in traps during May and June 2006. Blood and feathers were collected as described by Welsh (2008).

Minto Flats

Wigeon, teals, mallards, pintails, and shovelers were live-captured in traps at Minto Flats in cooperation with Alaska Department of Fish and Game banding operations during August 2006. Blood and feathers were collected as described by Welsh (2008). Briefly, 0.5 to 1.5 mL of blood were collected from the brachial or jugular vein, transferred to 2 mL EDTA tubes (BD Vacutainer), and stored at -80 °C. During

September 2006 and 2007, blood was collected post-mortem via heart puncture from hunter-killed ducks. The blood samples were stored in 2 mL EDTA tubes (BD Vacutainer) at -80 °C.

Determination of influenza infection status

In addition to blood and feather samples, cloacal swabs were taken from all ducks using methods described by Runstadler et al. (2007). RNA was extracted and used for the creation of cDNA for real-time RT-PCR (RRT-PCR) as previously described (Runstadler et al., 2007). The cDNA product was then screened for the avian influenza virus matrix gene using primers developed by Spackman et al. (2003) and modified by Runstadler et al. (2007). A threshold cutoff (C_t) value of <40 was used to assign infection status.

Chicken and duck Mx sequences and primer design

The NCBI Genbank database was searched in June 2007 for chicken and duck Mx mRNA sequences. The two available duck sequences (Genbank accession numbers Z21549 and Z21550) and a chicken sequence (Genbank accession number NM_204609) were aligned using the Clustalw algorithm in DNADynamo (Blue Tractor Software Ltd, UK). The area of duck sequence that best aligned with exon 13 of the chicken Mx mRNA was used to design primers in Primer3 (Rozen and Skaletsky, 2000). The forward (F) and reverse (R) primers are: Mx-F 5' – AGCAAGTAAACGCCTCTCCA – 3' and Mx-Rs 5' – GAAACTGGCAGTAAAGGTCAGC – 3'.

Genomic DNA extraction from whole blood and plucked body feathers

DNA was extracted from 5 - 10 µl of duck whole blood using the Qiagen Inc DNeasy Blood and Tissue Kit (Qiagen Inc., California, USA) according to the manufacturers' instructions. The DNeasy Blood and Tissue kit was also used to extract DNA from plucked body feathers as described by Bush et al. (2005). Briefly, one to six feather tips were placed in a 1.5 ml microfuge tube with 25 µl of 100 mg/ml DTT solution, 20 µl Proteinase K and 180 µl Buffer ATL. The mixture was incubated at 55 °C for 48 hours in a shaking incubator. After incubation, DNA was extracted according to the manufacturers' instructions regarding tissue. DNA concentration was quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Delaware, USA).

Mx gene amplification and sequencing

The Mx gene fragment was amplified through polymerase chain reaction (PCR) with the following reaction mixture: 80 ng/µl of genomic DNA, each primer at 0.4 µM, 1 unit Platinum Taq DNA polymerase (Invitrogen), 0.4 mM each dNTP, 2 mM $MgCl_2$, and 1X Platinum Taq polymerase buffer in a final volume of 25 µl. The PCR conditions were: 94 °C for 5 minutes followed by 35 cycles of 94 °C for 45 s, 56 °C for 45 s, 72 °C for 1 min, and a single incubation at 72 °C for 10 min followed by a hold at 4 °C. PCR products were run on a 1.5% agarose gel and then visualized using ethidium bromide staining. The expected PCR product size was approximately 340 bp. PCR products were cleaned with the QIAquick PCR purification kit (Qiagen Inc., California, USA) and sequenced with the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems; Foster City, CA) according to the manufacturers' recommendations. The sequencing

reactions were analyzed on an ABI 3100 sequencer at the University of Alaska Fairbanks DNA Core Facility and/or an ABI 3730XL sequencer at Macrogen Inc., Seoul, Korea.

DNA sequence alignment and analysis

Chromatograms were edited using Sequencher 4.7 (Gene Codes Corporation, USA), sequences aligned with the MUSCLE alignment algorithm (Edgar, 2004), and allelic phase was determined using PHASE v.2 (Stephens et al., 2001). Results from PHASE were verified by cloning and sequencing 50 heterozygous individuals across all five species. Diversity statistics including π (Tajima, 1983) and Tajima's D (Tajima, 1989) were estimated using DnaSp v 4.50 (Rozas and Rozas, 1999) and SITES (Hey and Wakeley, 1997). OmegaMap was used to search for evidence of diversifying selection within species (Wilson, 2005). Codon-based models in MEGA4.1 (Tamura et al., 2007) were also used to test for evidence of selection within species. Unrooted median-joining haplotype networks were constructed with Network v 4.5.1.0 in order to visualize how alleles were related within and between species (Bandelt et al., 1999). In order to evaluate how genetic variation was partitioned within and among species, an analysis of molecular variance (AMOVA) was performed using Arlequin v 3.11 (Excoffier et al., 2005).

Amino acid translation and analysis

The coding region was translated using web-based EMBOSS Transeq (http://bioweb2.pasteur.fr/docs/EMBOSS/transeq.html) and the variable sites were located using FaBox (http://www.birc.au.dk/~biopv/php/fabox/). SIFT was used to

predict the effects of amino acid substitutions on the functionality of the protein (Ng, 2003).

Disease association analysis

The number of influenza-positive and negative individuals in each haplotype was counted using a Perl script. In order to assess whether infection status was significantly associated with Mx haplotype, the program CLUMP was used (Sham and Curtis, 1995). A set of 100,000 simulations was run for each species and all species combined with both nucleotide and protein haplotypes. The p-value for the normal chi-squared (T1) table was used to determine statistical significance.

RESULTS

Nucleotide diversity

We sequenced a 336 base pair (bp) region of the duck Mx gene which encompasses exon 13 and a portion of the 3' UTR. Our sample consisted of 353 individuals (mean = 71, range = 15 - 204) from five species. The total number of sequences (n) and haplotypes (H) from each species can be seen in Table 2.

Mean pair-wise differences per site (π/bp) varied widely among species (Fig. 1). The greatest overall diversity (π_{total}) was found in teals (0.0061). Teals also had the highest π for nonsynonymous sites (π_s; 0.0078). Wigeons had the highest π for both synonymous sites (π_n; 0.0198) and silent sites (synonymous and noncoding sites; π_{silent}:

0.0097). Mallards were the least diverse in all categories with the exception of noncoding sites ($\pi_{noncoding}$: 0.0026).

Selection

We did not find evidence of long-term positive selection on our region of interest in any of the species studied. All species had dN/dS ratio less than one (more synonymous mutations than nonsynonymous mutations; Table 2). Mallards showed a significantly negative Tajima's D value while all other species had non-significant values (Table 2). No significant posterior probabilities of positive selection were found using omegaMap (data not shown).

Nucleotide haplotype network and disease association

Constructing a haplotype network allowed us to visualize the relationship between the nucleotide sequences from the five duck species (Fig. 2). We found 61 haplotypes, 32 of which were represented by two or more sequences from sampled individuals. One haplotype accounted for 49% of all sequences and 83% of all mallard sequences. Overall, we observed a large degree of interdigitation and some sharing of sequences; two haplotypes were shared by two species and two haplotypes were shared by three or more species. With the exception of the four shared haplotypes, sequences grouped by species. 82% of observed variation was explained by differences among species (AMOVA; $p < 0.00001$).

We used CLUMP to evaluate whether a statistical association exists between Mx allele and influenza infection status. Northern shovelers showed a marginally significant association ($p = 0.09$; Table 3). Five unique alleles contributed to this association.

Amino acid diversity

The coding region translated to 76 amino acids. We found 11 nonsynonymous substitutions and one stop codon; four substitutions, including the stop codon, were predicted to affect protein function based on sequence homology and the physical properties of amino acids (Fig. 4). Relative to the entire protein, the substitutions were E682STOP, L701F, S702G, and D717A. The L701F and S702G substitutions only occurred in teals; L701F appeared in four teal sequences, but S702G was present in 79% (41/52) of all teal sequences. The majority (92%; 24/26) of teal individuals was homozygous at position 702 and both individuals exhibiting the L701F substitution were homozygotes. The stop codon and D718A substitution each occurred in one mallard sequence. Overall, there were 17 unique protein sequences among the five duck species, nine of those were singletons, four were shared by multiple species, and five were only present in the teal population (Fig. 3).

DISCUSSION

We examined diversity and tested for evidence of selective pressure in a region of the Mx gene in five duck species: American green-winged teal, American wigeon, mallard, northern pintail, and northern shoveler. Additionally, we investigated whether an association exists between influenza infection status and Mx haplotype in these species.

Waterfowl appear resistant to the pathogenic effects of influenza virus yet may shed virus at high levels. Innate immune mechanisms, like that of Mx, may play a major role in limiting infection in ducks like that seen in other species (Lindenmann, 1962; Pavlovic et al., 1990; Ko et al., 2002; Nakajima et al., 2007).

A high degree of variability exists among duck species at the Mx locus; total nucleotide diversity (π_{total}) per site ranges from 0.0014 in mallards to 0.0061 in teals (Fig. 1). In contrast, Worley et al. (2008) examined diversity at MHC loci (BF: class I and BLB: class II) in red jungle fowl and domestic chicken. Mean π_{total} for red jungle fowl at the BF locus was 0.068 and 0.101 at the BLB locus. MHC loci must exhibit a vast array of diversity in order to effectively bind and present pathogen-derived peptides. Although Mx proteins do directly interact with viral components, they do not have to maintain as high diversity as that found at MHC loci because Mx proteins bind to more conserved regions such as the viral nucleoprotein or nuclear localization signal and to a more limited set of pathogens.

A more appropriate comparison would be between Mx proteins and toll-like receptor (TLR) proteins, as both show conservation across vertebrate species but diversity is needed in order to interact with a wide array of pathogens. In this study, there were 61 Mx haplotypes across 353 individuals. Palermo et al. (2009) explored variation at the TLR4 locus in 259 pigs and found 74 haplotypes and a total nucleotide diversity of 0.00077. The haplotype diversity is similar to that observed at the duck Mx locus, but there is less nucleotide diversity. This difference is diversity may be because mammalian

TLR4 recognizes bacterial lipopolysaccharide and extensive variation at this locus may significantly alter recognition and signal transduction (Palermo et al., 2009). Allelic diversity at TLR loci in humans is heterogeneous; in 100 individuals the number of alleles ranged from four to 15 across nine TLR genes. Several studies in humans, pigs, and cows have investigated single nucleotide polymorphisms (SNPs) at multiple TLR loci. It is difficult to directly compare those measures of diversity with those of the current study as our region is small relative to the size of TLR genes.

Constructing a haplotype network is a useful tool to visualize diversity and sequence relatedness. Figure 2 represents Mx nucleotide sequences from our five duck species and it is clear that, with a few exceptions, the sequences are grouped by species. There are four shared alleles, which could be an artifact of hybridization events or ancestral polymorphisms that are still segregating in these populations.

The low dN/dS ratios, non-positive values of Tajima's D (Table 2), and non-significant results from omegaMap did not show support of balancing or diversifying selective pressure on our region of interest. In addition, the haplotype network did not show patterns of balancing or diversifying selection. If strong selective pressure from disease or some other force was acting on this region, we might expect to see an advantageous allele at high frequency as a result of a selective sweep or perhaps a few alleles being maintained at stable frequencies driven by balancing selection. Although we did not measure diversity at the Mx locus relative to other genes, our summary statistics did not provide evidence for a selective sweep.

Berlin et al. (2008) studied Mx sequences from a small group of 14 mallards and did not find indicators of selection in exon 13 or the 3' UTR. However, when Berlin et al. (2008) considered the whole gene, avian *Mx* did show signatures of positive selection. The majority of positively selected sites were in what the authors designated the N-terminal "avian-specific region." The significance of this region is unknown, but it could be important to avian Mx expression or protein function, and would be interesting to examine in wild birds. The "avian-specific region" and our study region are separated by over 2000 bp, a distance which may have permitted enough recombination to obscure signatures of selection in the region we sequenced.

Given the lack of evidence for selective pressure on our region of interest, it is curious that we discovered a marginally significant association between Mx haplotype and infection status in shovelers ($p = 0.09$; Table 3). Shovelers are unique among the species in our study due to their specialized bill morphology (dense lamellae) that allows them to feed on small invertebrates filtered out of the water column (Dubowy, 1985). Shovelers devote around 80% of their foraging time to straining, while other dabbling ducks feed in the mud or around vegetation (Dubowy, 1985). Garamszegi and Møller (2007) found that species which employ surface-feeding foraging methods have a higher prevalence of influenza infection and Hill et al. (2009) discovered that lamellar density was significantly correlated with influenza prevalence. Northern shovelers' feeding strategy may lead them to interact with recently shed influenza virus more frequently than other dabbling ducks and perhaps this fact may imply a more important role for Mx than in other species.

Disease-related associations with the Mx gene have been found in other species as well. Nakajima et al. (2007) reported a porcine *Mx1* allele that does not confer resistance to influenza infection as the wild type does. In chickens, three SNPs in exon 13 were found to be significantly associated with commercial traits; the SNP previously described to be important in resistance or susceptibility to influenza infection was positively associated with increased antibody titers to vaccination for infectious bursal disease virus (Livant et al., 2007). Additionally, Mx genotype was also associated with differential antibody titers to influenza virus vaccination in chickens (Qu et al., 2009), suggesting that innate immune responses mediated by Mx in chickens, and possibly ducks, are tied to an integrated immune response to the virus. The association seen in our study is not without precedent and warrants further investigation. It is most likely a part of the Mx gene outside of our sequenced region that is driving the association in shovelers and a fine-mapping study of this chromosomal area in ducks with the development of genetic markers may help focus the true association.

Mallards also showed unique features among the species in our study. Total, silent, and nonsynonymous site diversity were lowest in mallards. Parker et al. (1981) also reported low genetic diversity across 20 loci in a wintering population of mallards, in spite of their large population size and ability to hybridize with other duck species. Berlin et al. (2008) studied eight exons (960 bp) of the Mx gene in 14 mallards and reported π_{syn} (0.0080) and π_{nonsyn} (0.0021). Our values of π_{syn} and π_{nonsyn} in mallards were lower than those reported by Berlin et al. which is most likely a reflection of higher conservation in our region of interest relative to the rest of the gene.

The relative lack of diversity in mallards in our study is illustrated by the largest circle in Fig. 2 which indicates that one haplotype represents 83% (342/410) of mallard Mx sequences. The other four species, in contrast, have several large clusters of alleles (Fig. 2) and greater total, silent, and nonsynonymous diversity (Fig. 1). Low genetic diversity and negative Tajima's D in mallards might be the result of a recent selective sweep, wherein strong positive selection has reduced genetic diversity at this locus. This would suggest that the major Mx allele present in mallards confers fitness and may actually be co-adapted to tolerate or control influenza infection better than alleles present in other species. In this case, we would expect a greater fitness effect of infection on populations of species besides mallards. Those fitness effects have only recently begun to be addressed (van Gils et al., 2007; Latorre-Margalef et al., 2009) and more work will shed light on the relative effects of infection in different dabbling duck species.

Alternatively, low diversity in mallards may be due to a recent population expansion, which is supported by a significantly negative Tajima's D (Table 2). In the context of influenza virus infection, our mallard data support a hypothesis that genetic diversification is important for maintaining immune response that prevents detectable infection from specific pathogens. Mallards are found to be positive for influenza virus more often than other duck species (Olsen et al., 2006) and this could be partly due to low genetic diversity at immune-related loci and an inability to resist infection on a population level.

We examined the predicted amino acid sequences from the five duck species to investigate diversity at the protein level. Nine of the 17 unique protein sequences were rare variants, only represented by one sequence. The four high-frequency sequences were shared by one or more species, but five sequences were present only in the teal population (Fig. 3). Three substitutions and one stop codon were predicted to affect protein function (boxed residues, Fig. 4). Two of those substitutions, L701F and S702G, were present only in the teal sequences. It is interesting to note that these substitutions occur at adjacent positions in the amino acid sequence (Fig. 4).

Without fully understanding the function of the amplified region in duck Mx protein, it is difficult to predict the effect of amino acid substitutions. However, we propose that the S702G mutation is not a deleterious substitution as it appears in 79% of teal sequences and involves substituting small, uncharged glycine for the larger, uncharged serine. There is a possible loss of a hydrogen bond, but theoretically there is no additional steric hindrance as result of the substitution. The characteristics of the substituted amino acid and the high frequency of the mutation suggest there could be an advantage for teals to have the S702G substitution. Steric interactions would appear to be more of an issue in the L701F substitution wherein a large aromatic ring is inserted in place of a branched hydrocarbon side chain. Four sequences contain the L701F substitution, all of which come from two teal individuals homozygous at that position.

This is the first study to characterize the Mx locus in a large number of individuals from multiple wild duck species. Our results demonstrate that nucleotide

diversity at this locus is being maintained at different levels in five duck species. We examined the 3' region of this locus because of its purported functional role, but other regions of the Mx gene may play important species-specific roles as well. In particular, studying diversity and selection in the avian-specific region documented by Berlin et al. (2008) could provide valuable insight into its functional importance. Mx gene expression in ducks in response to viral infection may provide clues to its relative importance in the host-virus interaction, as would investigation of a possible link between the Mx gene and antibody production. An assay of Mx protein antiviral activity in wild ducks in response to influenza and other viruses is also a necessary step.

In this study, we found 17 unique protein sequences with 11 variable sites translated from 336 bp of the Mx locus. Four of those mutations were predicted to affect protein function, but it is difficult to extend that prediction without a more complete understanding of the duck Mx protein. Our results demonstrate a variety of Mx proteins exist in wild ducks and we found evidence in northern shoveler populations of a possible influenza disease association with the Mx locus. More work clarifying the role of Mx and immune response in these species may lead to insight on the virus pathogenicity in other species.

ACKNOWLEDGEMENTS

The authors would like to thank the field crews that collected the samples used in this study, T. Welsh for genomic DNA, N. Takebayashi and D. Wolf for invaluable assistance with data analysis, J. Peters and K. Hueffer for useful discussion and C.

Mulder and reviewers in BIOL 693 at the University of Alaska Fairbanks for greatly improving this manuscript. DD was supported by an Institute of Arctic Biology Research Fellowship and an Alaska INBRE Graduate Fellowship. The project described was supported in part by INBRE Grant Number 5P20RR016466 from the National Center for Research Resources (NCRR); and in part by Sub-Award Contract from NIAID Number HHSN266200700009C, a component of the National Institutes of Health (NIH) and its contents are solely the responsibility of the authors and do not necessarily represent the official views of NCRR, NIAID or NIH.

REFERENCES

Arnheiter, H., Haller, O., Lindenmann, J., 1980. Host gene influence on interferon action in adult mouse hepatocytes: specificity for influenza virus. Virology. 1, 11-20.

Bacon, L. D., Hunter, D. B., Zhang, H. M., Brand, K., Etches, R., 2004. Retrospective evidence that the MHC (B haplotype) of chickens influences genetic resistance to attenuated infectious bronchitis vaccine strains in chickens. Avian Pathol. 6, 605-9.

Bandelt, H. J., Forster, P., Röhl, A., 1999. Median-joining networks for inferring intraspecific phylogenies. Mol Biol Evol. 1, 37-48.

Bazzigher, L., Schwarz, A., Staeheli, P., 1993. No enhanced influenza virus resistance of murine and avian cells expressing cloned duck Mx protein. Virology. 1, 100-12.

Benfield, C., Lyall, J., Kochs, G., Tiley, L., 2008. Asparagine 631 variants of the chicken Mx protein do not inhibit influenza virus replication in primary chicken embryo fibroblasts or in vitro surrogate assays. Journal of Virology. 15, 7533-7539.

Berlin, S., Qu, L., Li, X., Yang, N., Ellegren, H., 2008. Positive diversifying selection in avian Mx genes. Immunogenetics. 11, 689-697.

Bernasconi, D., Schultz, U., Staeheli, P., 1995. The interferon-induced Mx protein of chickens lacks antiviral activity. J Interferon Cytokine Res. 1, 47-53.

Bush, K., Vinsky, M., Aldridge, C., Paszkowski, C., 2005. A comparison of sample types varying in invasiveness for use in DNA sex determination in an endangered population of greater Sage-Grouse (Centrocercus uropihasianus). Conserv Genet. 5, 867-870.

Cardona, C., Xing, Z., Sandrock, C. E., Davis, C. E., 2009. Avian influenza in birds and mammals. Comparative Immunology, Microbiology and Infectious Diseases. 4, 255-73.

Clausen, D., Robus, M., Matthews, M. 1992. Minto Flats State Game Refuge Management Plan. Alaska Department of Fish and Game.

Dubowy, P., 1985. Feeding ecology and behavior of postbreeding male blue-winged teal and northern shovelers. Can J Zool. 6, 1292-1297.

Edgar, R. C., 2004. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics. 113.

Engelhardt, O., 2001. Interferon-induced antiviral Mx1 GTPase is associated with components of the SUMO-1 system and promyelocytic leukemia protein nuclear bodies. Exp. Cell Res. 2, 286-295.

Engelhardt, O., 2004. Mx1 GTPase accumulates in distinct nuclear domains and inhibits influenza A virus in cells that lack promyelocytic leukaemia protein nuclear bodies. Journal of General Virology. 8, 2315-2326.

Excoffier, L., Laval, G., Schneider, S., 2005. Arlequin (version 3.0): An integrated software package for population genetics data analysis. Evol Bioinform Online. 47-50.

Garamszegi, L. Z., Møller, A. P., 2007. Prevalence of avian influenza and host ecology. Proc Biol Sci. 1621, 2003-12.

Haller, O., Kochs, G., 2002. Interferon-induced mx proteins: dynamin-like GTPases with antiviral activity. Traffic. 10, 710-7.

Haller, O., Kochs, G., Weber, F., 2007. Interferon, Mx, and viral countermeasures. Cytokine Growth Factor Rev. 5-6, 425-433.

Haller, O., Staeheli, P., Kochs, G., 2008. Protective role of interferon-induced Mx GTPases against influenza viruses Rev. sci. tech. Off. int. Epiz. 1, 219-231.

Hey, J., Wakeley, J., 1997. A coalescent estimator of the population recombination rate. Genetics. 3, 833-46.

Hill, N. J., Takekawa, J. Y., Cardona, C. J., Ackerman, J. T., Schultz, A. K., Spragens, K. A., Boyce, W. M., 2009. Waterfowl ecology and avian influenza in California: do host traits inform us about viral occurrence? Avian Dis. 000-000.

Ito, T., Okazaki, K., Kawaoka, Y., Takada, A., Webster, R. G., Kida, H., 1995. Perpetuation of influenza A viruses in Alaskan waterfowl reservoirs. Archives of Virology. 7, 1163-72.

Janzen, C., Kochs, G., Haller, O., 2000. A monomeric GTPase-negative MxA mutant with antiviral activity. Journal of Virology. 17, 8202-6.

Kida, H., Yanagawa, R., Matsuoka, Y., 1980. Duck influenza lacking evidence of disease signs and immune response. Infect Immun. 2, 547-53.

Ko, J., Takada, A., Mitsuhashi, T., Agui, T., Watanabe, T., 2004. Native antiviral specificity of chicken Mx protein depends on amino acid variation at position 631. Animal Genetics. 2, 119-122.

Ko, J. H., Jin, H. K., Asano, A., Takada, A., Ninomiya, A., Kida, H., Hokiyama, H., Ohara, M., Tsuzuki, M., Nishibori, M., Mizutani, M., Watanabe, T., 2002. Polymorphisms and the differential antiviral activity of the chicken Mx gene. Genome Res. 4, 595-601.

Kochs, G., 2002. Self-assembly of human MxA GTPase into highly ordered dynamin-like oligomers. Journal of Biological Chemistry. 16, 14172-14176.

Kochs, G., Haller, O., 1999. Interferon-induced human MxA GTPase blocks nuclear import of Thogoto virus nucleocapsids. Proc Natl Acad Sci USA. 5, 2082.

Kochs, G., Janzen, C., Hohenberg, H., Haller, O., 2002. Antivirally active MxA protein sequesters La Crosse virus nucleocapsid protein into perinuclear complexes. Proceedings of the National Academy of Sciences. 5, 3153.

Latorre-Margalef, N., Gunnarsson, G., Munster, V. J., Fouchier, R. A. M., Osterhaus, A. D. M. E., Elmberg, J., Olsen, B., Wallensten, A., Haemig, P. D., Fransson, T., Brudin, L., Waldenström, J., 2009. Effects of influenza A virus infection on migrating mallard ducks. Proc Biol Sci. 1659, 1029-36.

Lindenmann, J., 1962. Resistance of mice to mouse-adapted influenza A virus. Virology. 203-4.

Lindenmann, J., Klein, P., 1966. Further studies on the resistance of mice to myxoviruses. Archives of Virology. 1, 1-12.

Livant, E., Brigati, J., Ewald, S., 2004. Diversity and locus specificity of chicken MHC B class I sequences. Animal Genetics. 1, 18-27.

Livant, E., Avendano, S., Mcleod, S., Ye, X., Lamont, S., Dekkers, J., Ewald, S., 2007. Mx1 exon 13 polymorphisms in broiler breeder chickens and associations with commercial traits. Animal Genetics. 2, 177-179.

Meier, E., Fäh, J., Grob, M. S., End, R., Staeheli, P., Haller, O., 1988. A family of interferon-induced Mx-related mRNAs encodes cytoplasmic and nuclear proteins in rat cells. Journal of Virology. 7, 2386-93.

Nakajima, E., Morozumi, T., Tsukamoto, K., Watanabe, T., Plastow, G., Mitsuhashi, T., 2007. A naturally occurring variant of porcine Mx1 associated with increased susceptibility to influenza virus in vitro. Biochem Genet. 1-2, 11-24.

Ng, A., Wang, J., Shaw, P., 2009. Structure and sequence analysis of influenza A virus nucleoprotein. Science in China Series C: Life Sciences.

Ng, P., 2003. SIFT: predicting amino acid changes that affect protein function. Nucleic Acids Research. 13, 3812-3814.

Olsen, B., Munster, V., Wallensten, A., Waldenström, J., Osterhaus, A. D., Fouchier, R. A., 2006. Global patterns of influenza A virus in wild birds. Science. 5772, 384-8.

Palermo, S., Capra, E., Torremorell, M., Dolzan, M., Davoli, R., Haley, C. S., Giuffra, E., 2009. Toll-like receptor 4 genetic diversity among pig populations. Animal Genetics. 3, 289-99.

Palm, M., Leroy, M., Thomas, A., Linden, A., Desmecht, D., 2007. Differential anti-influenza activity among allelic variants at the Sus scrofa Mx1 locus. Journal of Interferon & Cytokine Research. 2, 147-156.

Parker, L. E., Bolen, E. G., Baker, R. J., 1981. Genetic variation in a winter population of mallard ducks. Southwest Nat. 4, 425-428.

Pavlovic, J., Zurcher, T., Haller, O., Staeheli, P., 1990. Resistance to influenza virus and vesicular stomatitis virus conferred by expression of human MxA protein. The Journal of Virology. 7, 3370.

Petrula, M. J. 1994. M.S. Thesis. University of Alaska Fairbanks.

Qu, L. J., Li, X. Y., Xu, G. Y., Ning, Z. H., Yang, N., 2009. Lower antibody response in chickens homozygous for the Mx resistant allele to avian influenza. Asian Austral J Anim. 4, 465-470.

Rozas, J., Rozas, R., 1999. DnaSP version 3: an integrated program for molecular population genetics and molecular evolution analysis. Bioinformatics. 2, 174-5.

Rozen, S., Skaletsky, H., 2000. Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol. 365-86.

Runstadler, J., Happ, G., Slemons, R., Sheng, Z., Gundlach, N., Petrula, M., Senne, D., Nolting, J., Evers, D., Modrell, A., Huson, H., Hills, S., Rothe, T., Marr, T., Taubenberger, J., 2007. Using RRT-PCR analysis and virus isolation to determine the prevalence of avian influenza virus infections in ducks at Minto Flats State Game Refuge, Alaska, during August 2005. Archives of Virology. 10, 1901-1910.

Sadler, A., Williams, B., 2008. Interferon-inducible antiviral effectors. Nat Rev Immunol. 7, 559-568.

Sham, P. C., Curtis, D., 1995. Monte Carlo tests for associations between disease and alleles at highly polymorphic loci. Ann Hum Genet. Pt 1, 97-105.

Spackman, E., Senne, D. A., Bulaga, L. L., Myers, T. J., Perdue, M. L., Garber, L. P., Lohman, K., Daum, L. T., Suarez, D. L., 2003. Development of real-time RT-PCR for the detection of avian influenza virus. Avian Dis. 3 Suppl, 1079-82.

Stephens, M., Smith, N. J., Donnelly, P., 2001. A new statistical method for haplotype reconstruction from population data. Am J Hum Genet. 4, 978-89.

Stertz, S., Reichelt, M., Krijnse-Locker, J., Mackenzie, J., Simpson, J. C., Haller, O., Kochs, G., 2006. Interferon-induced, antiviral human MxA protein localizes to a distinct subcompartment of the smooth endoplasmic reticulum. J Interferon Cytokine Res. 9, 650-60.

Tajima, F., 1983. Evolutionary relationship of DNA sequences in finite populations. Genetics. 2, 437-60.

Tajima, F., 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics. 3, 585-95.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0. Mol Biol Evol. 8, 1596-1599.

van Gils, J. A., Munster, V. J., Radersma, R., Liefhebber, D., Fouchier, R. A. M., Klaassen, M., 2007. Hampered foraging and migratory performance in swans infected with low-pathogenic avian influenza A virus. PLoS ONE. 1, e184.

Webster, R. G., Bean, W. J., Gorman, O. T., Chambers, T. M., Kawaoka, Y., 1992. Evolution and ecology of influenza A viruses. Microbiol Rev. 1, 152-79.

Welsh, T. 2008. M.S. Thesis. University of Alaska Fairbanks.

Wilson, D., 2005. Estimating diversifying selection and functional constraint in the presence of recombination. Genetics. 3, 1411-1425.

Worley, K., Gillingham, M., Jensen, P., Kennedy, L., Pizzari, T., Kaufman, J., Richardson, D., 2008. Single locus typing of MHC class I and class II B loci in a population of red jungle fowl. Immunogenetics. 5, 233-247.

Zürcher, T., Pavlovic, J., Staeheli, P., 1992. Mechanism of human MxA protein action: variants with changed antiviral properties. EMBO J. 4, 1657-61.

Table 1 - Summary of samples from five species of ducks. Genomic DNA samples were from an archived collection originally extracted from blood and feathers (Welsh, 2008). Additional feathers and blood were processed as described in the text.

Species	**n**	**Sample type**	**Location**	**Year**
American green-winged teal	13	Genomic DNA	Minto Flats	2006
(*Anas crecca carolinensis)*	1	Feathers	Minto Flats	2006
	12	Blood	Minto Flats	2007
Total	**26**			
American wigeon	5	Genomic DNA	Yukon Flats	2006
(*Anas americana)*	10	Blood	Minto Flats	2007
Total	**15**			
Mallard	140	Genomic DNA	Minto Flats	2006
(*Anas platyrynchos)*	11	Feathers	Minto Flats	2006
	53	Blood	Minto Flats	2007
Total	**204**			
Northern pintail	34	Genomic DNA	Minto Flats	2006
(*Anas acuta)*	5	Feathers	Minto Flats	2006
	26	Blood	Minto Flats	2007
Total	**65**			
Northern shoveler	7	Genomic DNA	Yukon Flats	2006
(*Anas clypeata)*	36	Blood	Minto Flats	2007
Total	**43**			

Table 2 - Summary statistics for Mx exon 13 and 3' UTR from five duck species

Species	n[a]	H	S	D_{Taj}	dN/dS
American green-winged teal (*Anas crecca carolinensis*)	52	9	8	0.41	0.86
American wigeon (*Anas americana*)	30	9	12	-1.41	0.06
Mallard (*Anas platyrynchos*)	408	24	22	-2.22*	0.19
Northern pintail (*Anas acuta*)	130	17	16	-1.35	0.84
Northern shoveler (*Anas clypeata*)	86	9	9	-1.24	0.47

[a] n, number of alleles sampled; H, number of haplotypes; S, number of segregating sites; D_{Taj}, Tajima's D; dN/dS, the ratio of π_{nonsyn} to π_{syn}; *P < 0.01

Table 3 - Significance values for disease association. Nucleotide sequences from five species of wild ducks were tested for an association between influenza infection state and Mx haplotype using CLUMP.

Species	**Nucleotide p-value**
American green-winged teal (*Anas crecca carolinensis*)	0.20
American wigeon (*Anas americana*)	0.23
Mallard (*Anas platyrynchos*)	0.27
Northern pintail (*Anas acuta*)	0.29
Northern shoveler (*Anas clypeata)*	0.09
All species	0.16

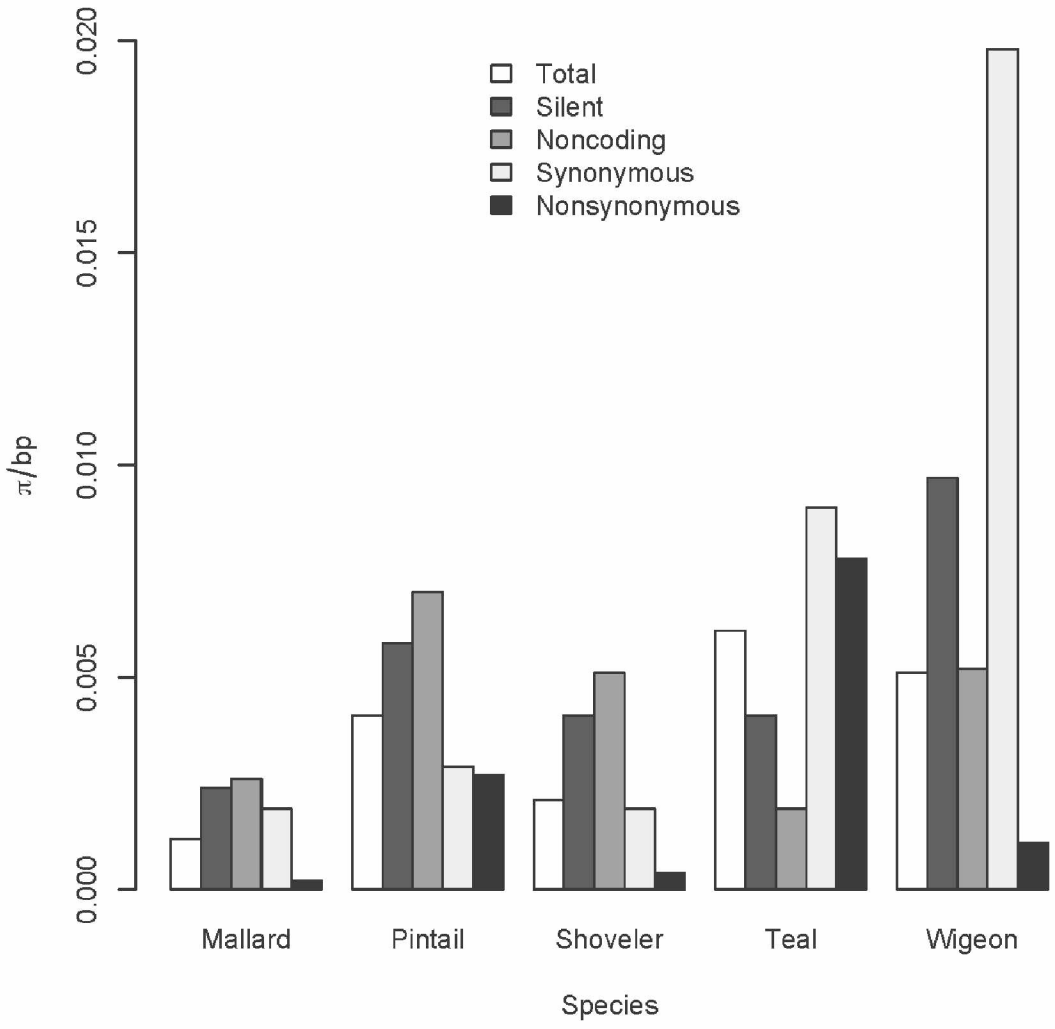

Figure 1 - Mean pairwise differences (π) per base pair (bp) for exon 13 and 3' UTR of the Mx gene in five duck species.

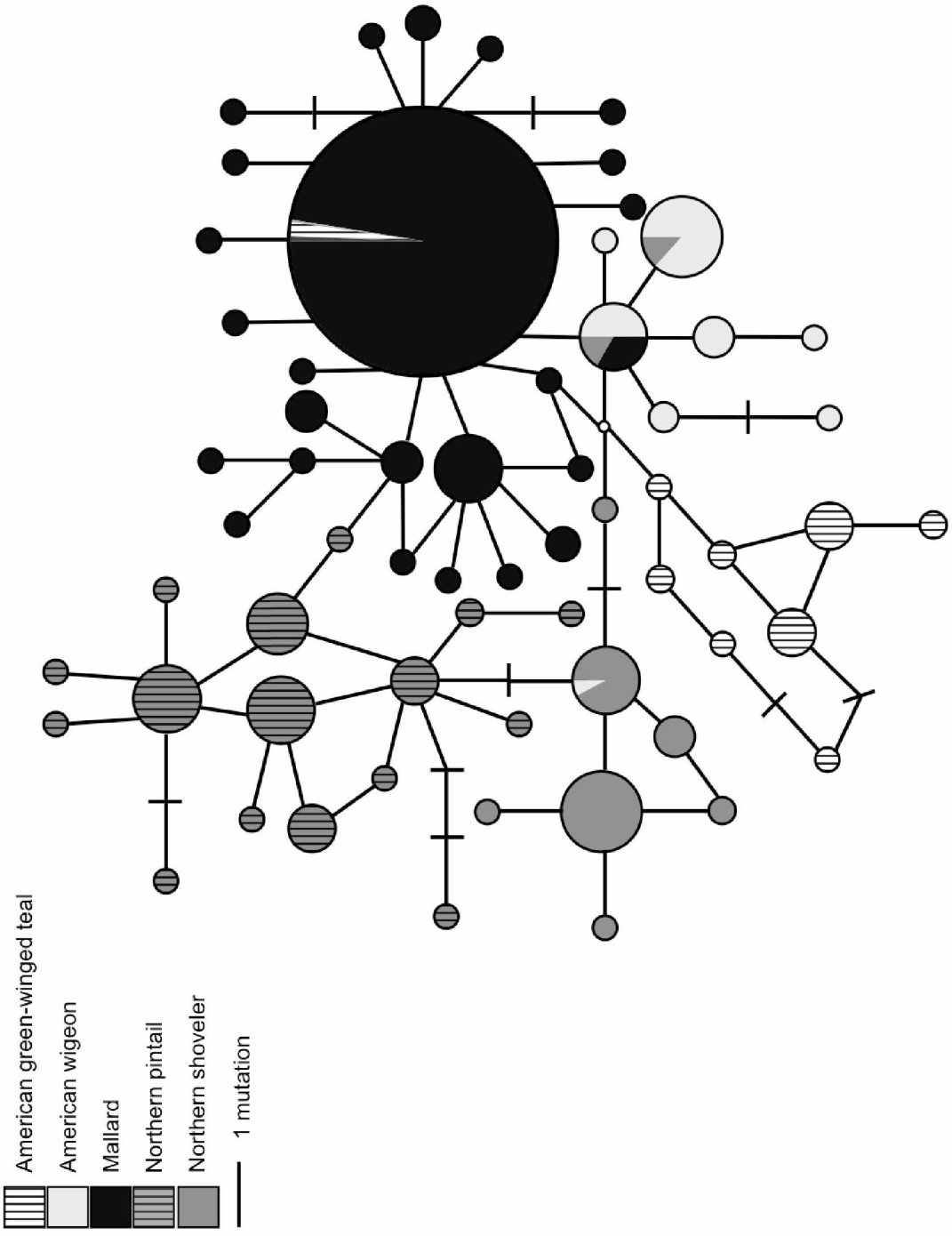

Figure 2 - Unrooted median joining network showing the relationship among Mx nucleotide sequences (336 bp) from five duck species. Species are denoted in the legend. The area of circles is proportional to the number of sequences in that haplotype.

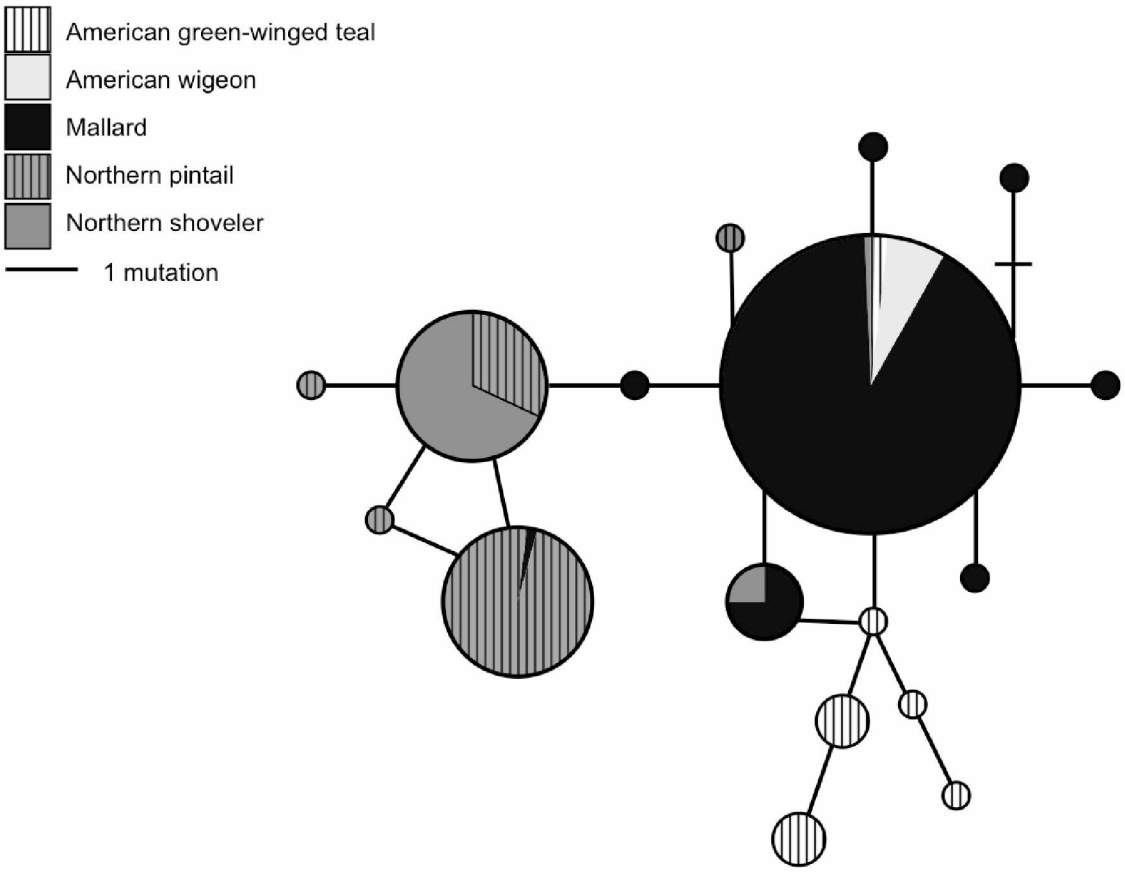

Figure 3 - Unrooted median joining network showing the relationship among Mx protein sequences (76 amino acids) from five duck species. Species are denoted in the legend. The area of circles is proportional to the number of sequences in that haplotype.

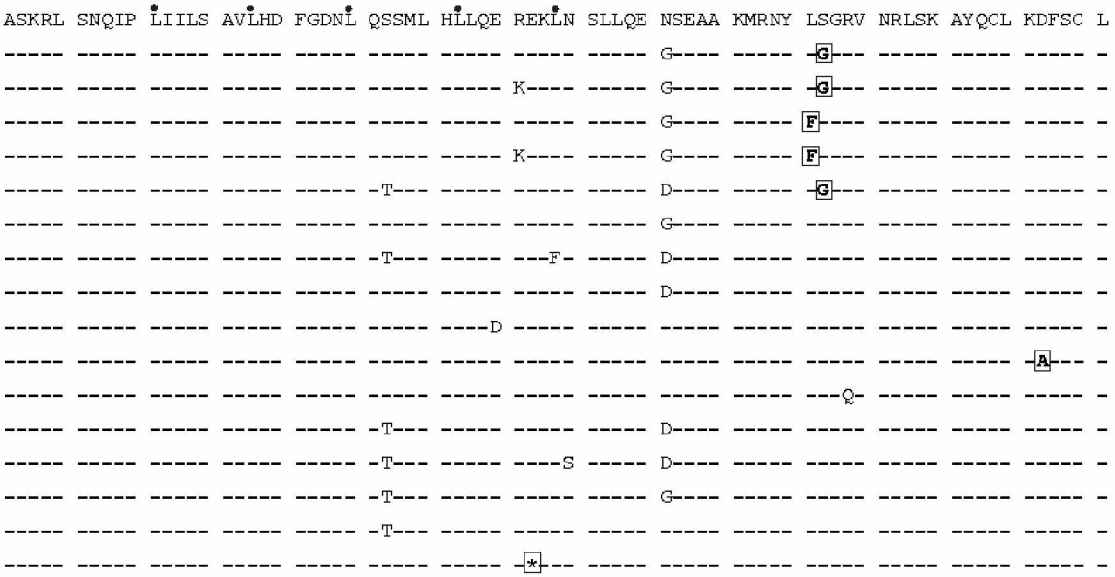

Figure 4 - Unique Mx protein amino acid sequences (pos 646 – 721). Dashed lines indicate residues are identical to the top sequence. Boxed residues are predicted to affect protein function. Dots denote a leucine zipper motif.

CONCLUSION

There are few studies investigating the innate immune system in wild populations, as it can be expensive and logistically difficult when compared to model organisms. In particular, little is known about the immunogenetics of dabbling ducks and how genetic diversity at immune-related loci may impact their role in the ecology of influenza virus. Susceptibility or resistance to infection is likely to be impacted by the genetic background of the host, but genetic variation in resistance genes has not been extensively studied in the context of influenza virus. Data from large-scale human studies have suggested some likelihood of genetic predisposition towards susceptibility to influenza infection, but risks from exposure or behavior were not accounted for (Trammell and Toth, 2008). Inbred mouse lines with known genotypes have provided insight into the immunology of influenza virus infection, which helps identify candidate genes to study in other organisms. However, it is unknown to what extent these findings can be extended to wild populations.

This study attempted to address the deficiency of knowledge regarding immunogenetics in wild populations using the Mx gene, a gene that may play a role in resistance to influenza virus. If there is coevolution between the virus and resistance genes such as Mx, we may expect to see evidence of positive selection in the Mx gene. A 336 bp region of the duck Mx gene was characterized in over 300 individuals, representing five wild duck species. We found a wide range of variation among the species studied, but did not find evidence of positive selection. However, we did find a

surprising, if marginal, statistical association between influenza infection status and Mx allele in northern shovelers.

Control of immune response is likely polygenic in most cases, but evaluating polymorphism at one locus is a step towards understanding host genetics. In the region of the Mx gene studied here, we found that mallards maintained relatively low total nucleotide diversity and teals were the most diverse; shovelers, pintails and wigeons fell in between. Selective pressure does not appear to be directly acting on this region, but regional genetic diversity could be affected by selection acting on other parts of the gene.

Variation or a lack thereof at immune-related loci can indicate how a host and pathogen interact. There is an increasing body of knowledge concerning the genetic signatures left in both the host and pathogen genomes that can be indicative of host-pathogen coevolution. In their investigation of human MHC, Borghans et al. (2004) found the extensive variation present there is better explained by host-pathogen coevolution than either heterozygote advantage or frequency-dependent selection alone. Lobo et al. (2009) demonstrated that *Flaviviridae* viruses and the hosts they infect had a unique pattern of amino acid codon usage. Interestingly, single-host *Flaviviridae* viruses showed a codon usage pattern that was more similar to its host than to other members of *Flaviviridae*. Many factors control the generation and maintenance of genetic diversity, but interaction with pathogens, especially at immune-related loci, seems to be particularly important.

Obtaining the sequence of the entire duck Mx gene would allow us to expand this investigation of the level of conservation among wild duck species and see if evidence of selective pressure is also present, like that seen in a small sample of mallards (Berlin et al., 2008). Polymorphism at the Mx locus could affect the gene and protein product in a number of ways. Bazzigher et al. (1993) identified alternative sites where pre-translational modifications were made in the duck Mx gene that resulted in four distinct proteins. Changes to those sites would most likely affect protein translation. Mx gene expression is regulated by interferon (IFN) through IFN-stimulated response elements (ISREs) in the promoter. A single nucleotide polymorphism (SNP) in the promoter of human MxA confers a four-fold increase in gene expression in response to IFN (Fernández-Arcás et al., 2004). Finally, it has been demonstrated that single amino acid changes in the Mx protein can affect cellular localization, binding of GTP, viral target recognition, and self-assembly (Haller et al., 2009). Information from the complete duck Mx genetic sequence would allow us to evaluate whether the effects of polymorphism seen in other species are applicable to wild ducks as well.

Polymorphism at the Mx locus could also have downstream effects on the humoral immune system. A recent study connected the Mx gene with differential antibody production in chickens (Qu et al., 2009). This suggests the duck Mx gene could be involved in one or both arms of the immune response. To truly address innate immune activity, the antiviral activity of Mx proteins from wild avian species should be re-evaluated using low pathogenic avian influenza viruses relevant to the wild populations of dabbling ducks and these species. The duck antibody response to influenza infection is

poorly understood and investigating whether wild birds with known Mx genotypes exhibit a similar antibody response as seen in chickens could also clarify the link between humoral and innate immune responses in ducks.

Closing the gap in our knowledge of immune function in ducks is necessary to grasp the full interaction between the reservoir and the pathogen, influenza virus. Extensive research on murine models of influenza and genetics has been done, but as mice are not implicated as major vectors of the virus, it would be more appropriate to develop a deeper understanding of a reservoir species. In turn, this could aid in developing therapies or vaccination strategies for other species involved in influenza ecology: poultry, swine, and humans.

REFERENCES

Aebi, M., Fäh, J., Hurt, N., Samuel, C. E., Thomis, D., Bazzigher, L., Pavlovic, J., Haller, O., Staeheli, P., 1989. cDNA structures and regulation of two interferon-induced human Mx proteins. Mol Cell Biol. 11, 5062-72.

Bazzigher, L., Schwarz, A., Staeheli, P., 1993. No enhanced influenza virus resistance of murine and avian cells expressing cloned duck Mx protein. Virology. 1, 100-12.

Benfield, C., Lyall, J., Kochs, G., Tiley, L., 2008. Asparagine 631 variants of the chicken Mx protein do not inhibit influenza virus replication in primary chicken embryo fibroblasts or in vitro surrogate assays. Journal of Virology. 15, 7533-7539.

Berlin, S., Qu, L., Li, X., Yang, N., Ellegren, H., 2008. Positive diversifying selection in avian Mx genes. Immunogenetics. 11, 689-697.

Bernasconi, D., Schultz, U., Staeheli, P., 1995. The interferon-induced Mx protein of chickens lacks antiviral activity. J Interferon Cytokine Res. 1, 47-53.

Borghans, J. A. M., Beltman, J. B., De Boer, R. J., 2004. MHC polymorphism under host-pathogen coevolution. Immunogenetics. 11, 732-9.

Fernández-Arcás, N., Blanco, A., Gaitán, M. J., Nyqvist, M., Alonso, A., Reyes-Engel, A., 2004. Differential transcriptional expresión of the polymorphic myxovirus resistance protein A in response to interferon-alpha treatment. Pharmacogenetics. 3, 189-93.

Haller, O., Staeheli, P., Kochs, G., 2009. Protective role of interferon-induced Mx GTPases against influenza viruses. Rev - Off Int Epizoot. 1, 219-31.

Haller, O., Frese, M., Rost, D., Nuttall, P., Kochs, G., 1995. Tick-borne Thogoto virus infection in mice is inhibited by the orthomyxovirus resistance gene product Mx1. Journal of Virology. 4, 2596-2601.

Haller, O., Kochs, G., Weber, F., 2006. The interferon response circuit: Induction and suppression by pathogenic viruses. Virology. 1, 119-130.

Haller, O., Kochs, G., Weber, F., 2007a. Interferon, Mx, and viral countermeasures. Cytokine Growth Factor Rev. 5-6, 425-433.

Haller, O., Staeheli, P., Kochs, G., 2007b. Interferon-induced Mx proteins in antiviral host defense. Biochimie. 6-7, 812-818.

Haller, O., Stertz, S., Kochs, G., 2007c. The Mx GTPase family of interferon-induced antiviral proteins. Microb. Infect. 14-15, 1636-1643.

Haller, O., Staeheli, P., Kochs, G., 2009. Protective role of interferon-induced Mx GTPases against influenza viruses. Rev - Off Int Epizoot. 1, 219-31.

Hatchette, T. F., Walker, D., Johnson, C., Baker, A., Pryor, S. P., Webster, R. G., 2004. Influenza A viruses in feral Canadian ducks: extensive reassortment in nature. J Gen Virol. Pt 8, 2327-37.

Horisberger, M. A., 1992. Interferon-induced human protein MxA is a GTPase which binds transiently to cellular proteins. Journal of Virology. 8, 4705-9.

Hou, Z., Xu, G., Su, Z., Yang, N., 2007. Purifying selection and positive selection on the myxovirus resistance gene in mammals and chickens. Gene. 1, 188-195.

Ito, T., Okazaki, K., Kawaoka, Y., Takada, A., Webster, R. G., Kida, H., 1995. Perpetuation of influenza A viruses in Alaskan waterfowl reservoirs. Archives of Virology. 7, 1163-72.

Janzen, C., Kochs, G., Haller, O., 2000. A monomeric GTPase-negative MxA mutant with antiviral activity. Journal of Virology. 17, 8202-6.

Jin, H., Takada, A., Kon, Y., Haller, O., Watanabe, T., 1999. Identification of the murine Mx2 gene: interferon-induced expression of the Mx2 protein from the feral mouse gene confers resistance to vesicular stomatitis virus. Journal of Virology. 6, 4925.

Jin, H., Yamashita, T., Ochiai, K., Haller, O., Watanabe, T., 1998. Characterization and expression of the Mx1 gene in wild mouse species. Biochem Genet. 9, 311-322.

Jin, H. K., Yoshimatsu, K., Takada, A., Ogino, M., Asano, A., Arikawa, J., Watanabe, T., 2001. Mouse Mx2 protein inhibits hantavirus but not influenza virus replication. Archives of Virology. 1, 41-9.

Ko, J., Takada, A., Mitsuhashi, T., Agui, T., Watanabe, T., 2004. Native antiviral specificity of chicken Mx protein depends on amino acid variation at position 631. Animal Genetics. 2, 119-122.

Ko, J. H., Jin, H. K., Asano, A., Takada, A., Ninomiya, A., Kida, H., Hokiyama, H., Ohara, M., Tsuzuki, M., Nishibori, M., Mizutani, M., Watanabe, T., 2002. Polymorphisms and the differential antiviral activity of the chicken Mx gene. Genome Res. 4, 595-601.

Kochs, G., 2002. Self-assembly of human MxA GTPase into highly ordered dynamin-like oligomers. Journal of Biological Chemistry. 16, 14172-14176.

Kuchipudi, S. V., Nelli, R., White, G. A., Bain, M., Chang, K. C., Dunham, S., 2009. Differences in influenza virus receptors in chickens and ducks: Implications for interspecies transmission. J Mol Genet Med. 1, 143-51.

Li, X. Y., Qu, L. J., Hou, Z. C., Yao, J. F., Xu, G. Y., Yang, N., 2007. Genomic structure and diversity of the chicken Mx gene. Poult Sci. 4, 786-9.

Lindenmann, J., 1962. Resistance of mice to mouse-adapted influenza A virus. Virology. 203-4.

Lindenmann, J., 1964. Inheritance of resistance to influenza virus in mice. Proc Soc Exp Biol Med. 506-9.

Livant, E., Avendano, S., Mcleod, S., Ye, X., Lamont, S., Dekkers, J., Ewald, S., 2007. Mx1 exon 13 polymorphisms in broiler breeder chickens and associations with commercial traits. Animal Genetics. 2, 177-179.

Lobo, F. P., Mota, B. E. F., Pena, S. D. J., Azevedo, V., Macedo, A. M., Tauch, A., Machado, C. R., Franco, G. R., 2009. Virus-host coevolution: common patterns of nucleotide motif usage in Flaviviridae and their hosts. PLoS ONE. 7, e6282.

Meier, E., Fäh, J., Grob, M. S., End, R., Staeheli, P., Haller, O., 1988. A family of interferon-induced Mx-related mRNAs encodes cytoplasmic and nuclear proteins in rat cells. Journal of Virology. 7, 2386-93.

Mogensen, T. H., 2009. Pathogen recognition and inflammatory signaling in innate immune defenses. Clin Microbiol Rev. 2, 240-73.

Obenauer, J. C., Denson, J., Mehta, P. K., Su, X., Mukatira, S., Finkelstein, D. B., Xu, X., Wang, J., Ma, J., Fan, Y., Rakestraw, K. M., Webster, R. G., Hoffmann, E., Krauss, S., Zheng, J., Zhang, Z., Naeve, C. W., 2006. Large-scale sequence analysis of avian influenza isolates. Science. 5767, 1576-80.

Olsen, B., Munster, V., Wallensten, A., Waldenström, J., Osterhaus, A. D., Fouchier, R. A., 2006. Global patterns of influenza A virus in wild birds. Science. 5772, 384-8.

Palm, M., Leroy, M., Thomas, A., Linden, A., Desmecht, D., 2007. Differential anti-influenza activity among allelic variants at the Sus scrofa Mx1 locus. Journal of Interferon & Cytokine Research. 2, 147-156.

Pavlovic, J., Haller, O., Staeheli, P., 1992. Human and mouse Mx proteins inhibit different steps of the influenza virus multiplication cycle. Journal of Virology. 4, 2564-9.

Qu, L. J., Li, X. Y., Xu, G. Y., Ning, Z. H., Yang, N., 2009. Lower antibody response in chickens homozygous for the Mx resistant allele to avian influenza. Asian Austral J Anim. 4, 465-470.

Runstadler, J., Happ, G., Slemons, R., Sheng, Z., Gundlach, N., Petrula, M., Senne, D., Nolting, J., Evers, D., Modrell, A., Huson, H., Hills, S., Rothe, T., Marr, T., Taubenberger, J., 2007. Using RRT-PCR analysis and virus isolation to determine the prevalence of avian influenza virus infections in ducks at Minto Flats State Game Refuge, Alaska, during August 2005. Archives of Virology. 10, 1901-1910.

Sharp, G. B., Kawaoka, Y., Jones, D. J., Bean, W. J., Pryor, S. P., Hinshaw, V., Webster, R. G., 1997. Coinfection of wild ducks by influenza A viruses: distribution patterns and biological significance. Journal of Virology. 8, 6128-35.

Staeheli, P., Yu, Y. X., Grob, R., Haller, O., 1989. A double-stranded RNA-inducible fish gene homologous to the murine influenza virus resistance gene Mx. Mol Cell Biol. 7, 3117-21.

Trammell, R. A., Toth, L. A., 2008. Genetic susceptibility and resistance to influenza infection and disease in humans and mice. Expert Rev Mol Diagn. 4, 515-29.

Webster, R. G., Bean, W. J., Gorman, O. T., Chambers, T. M., Kawaoka, Y., 1992. Evolution and ecology of influenza A viruses. Microbiol Rev. 1, 152-79.

Webster, R. G., Krauss, S., Hulse-Post, D., Sturm-Ramirez, K., 2007. Evolution of influenza a viruses in wild birds. Journal of Wildlife Diseases. 3, S1-S6.

Zürcher, T., Pavlovic, J., Staeheli, P., 1992. Mouse Mx2 protein inhibits vesicular stomatitis virus but not influenza virus. Virology. 2, 796-800.