MYCOPLASMA GALLISEPTICUM INFECTION IN HOUSE FINCHES: VIRULENCE, IMMUNOGENICITY, AND CHRONIC DISEASE

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MYCOPLASMA GALLISEPTICUM INFECTION IN HOUSE FINCHES: VIRULENCE, IMMUNOGENICITY, AND CHRONIC DISEASE

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Mycoplasma gallisepticum (MG), a respiratory pathogen of poultry, was first documented as a cause of conjunctivitis in free-living house finches (*Haemorhous,* formerly *Carpodacus*, *mexicanus*) in 1994. This work explores virulence and immunogenicity of several isolates of MG collected from free-living house finches, and provides evidence that individual birds can have very different courses of disease when experimentally inoculated with the same dose of MG.

A quantitative PCR assay utilizing the *mgc2* gene was developed to quantify MG in conjunctival swab samples. To measure house finch lachrymal antibody response, a polyclonal reagent was developed to detect house finch IgA. A region of house finch IgA heavy chain was PCR-amplified from spleen cDNA, the heavy chain fragment was produced using a bacterial expression system, and rabbit anti-sera were generated against the recombinant protein.

House finches were inoculated with MG isolates Virginia (VA)1994, California (CA)2006, or North Carolina (NC)2006, which were isolated from free-living house finches with conjunctivitis in 1994, 2006, and 2006, respectively. Infection with NC2006 resulted in the most severe eye lesions, highest pathogen loads, and highest levels of MG-specific lachrymal and serum antibodies. Infection with CA2006 caused the least severe eye lesions, lowest pathogen load, and weakest antibody response. A small number of birds in each group developed protracted, severe disease in spite of robust antibody responses. Immunoblot analyses indicated

that isolates are antigenically similar, suggesting that there may be partial cross-protection if a house finch encounters two or more strains of MG throughout the course of its lifetime.

Chronic disease was further studied with experimental inoculations of house finches using VA1994 and North Carolina (NC1995, NC1996, NC2006, NC2008) field isolates. After inoculation, birds with chronic disease had significantly higher pathogen loads and antibody responses than did birds with acute disease. Using a monoclonal antibody specific for a variant of MG VlhA immunodominant surface protein, I show that VlhA expression differs among MG isolates studied, and that *in vivo* VlhA changes occur in house finches infected with MG. The results provide evidence that chronic MG disease has both pathogen-mediated and immunopathologic components.

BIOGRAPHICAL SKETCH

Jessica Lee Grodio was born in New York City and grew up just north of the city, in the Mid-Hudson Valley. Her love of birds developed through her interactions with many pets, including Rhode Island Red chickens, ducks, budgies, and an incredible cockatiel. As a child, she participated in the Cornell Laboratory of Ornithology's citizen science projects (Project Feeder Watch and the House Finch Disease Survey) and these activities further developed her early interest in birds.

Following graduation from Arlington High School in LaGrangeville, NY, Jessica attended Cornell University's College of Agriculture and Life Sciences with an interest in biology. After taking an undergraduate ornithology course, Jessica pursued field research on reproductive effort in tree swallows. She also began volunteering at the Cornell Hospital for Animals' wildlife clinic. Jessica enjoyed research and her work at the wildlife clinic, so she applied to Cornell's dual DVM/PhD program in hopes of combining her interests in veterinary medicine and ornithology. In 2006, she earned a B.S. in biological sciences with distinction in research.

After acceptance into the DVM/PhD program, she returned to Cornell in the fall of 2006. She joined Dr. Ton Schat's laboratory and worked with the Cornell Lab of Ornithology's house finch disease research group. During her veterinary studies, she enjoyed working at Cornell's Exotic Animal Service, Wildlife Health Center, and at the Rosamond-Gifford Zoo in Syracuse, NY. She also completed a clinical externship at the Wildlife Conservation Society in Bronx, NY. She received her DVM degree in 2012, and returned to the PhD program to finish her thesis.

Dedicated to
Sunny
John
Mom
&
Dad

"....They would not find me changed from him they knew–
Only more sure of all I thought was true."
-Robert Frost, 1913

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CHAPTER 1

Introduction

Bacteria of the genus *Mycoplasma* cause a range of diseases in both humans and veterinary species. These bacteria lack a cell wall, have tiny genomes, and several species are known for causing chronic infection in spite of a robust host immune response. In veterinary species, these bacteria are often incompletely cleared by antibiotic therapy, and hosts may still harbor the organism and may continue to have clinical signs during or after antibiotic therapy (e.g., Bebear and Kempf 2005, Donnelly 2004).

Mycoplasma gallisepticum (MG) is well known for causing acute and chronic respiratory disease in chickens and infectious sinusitis in turkeys (Ley 2003). In 1994, MG was documented in a novel host, the house finch (*Haemorhous*, formerly *Carpodacus*, *mexicanus*) (Ley et al. 1996). In this species, MG causes predominately conjunctivitis, with respiratory signs being much less common compared to infection in poultry. Since its introduction into free-living house finch populations, conjunctivitis and positive serum plate agglutination tests have less commonly been documented in other songbirds, most notably American goldfinches (*Carduelis tristis*) and purple finches (*Carpodacus purpureus*) (Hartup et al. 2000, Hartup et al. 2001).

MG infection in chickens has been subject to much research due to the major economic impact of the disease on the poultry industry globally. Strains of MG isolated from commercial poultry are quite variable with respect to pathogenicity, transmissibility, and immunogenicity in chickens and turkeys (Rodriguez and Kleven 1980, Sanei et al. 2007). While some variations at the genomic level have been documented among MG isolates from house finches (Pillai et al. 2003, Tulman et al. 2012), there has only been one report suggesting differences in pathogenicity

based on experimental infections under controlled conditions (Hawley et al. 2010). One goal of this work is to examine if circulating house finch "strains" of MG differ in pathogenicity and immunogenicity.

Additionally, previous studies (Kollias et al. 2004, Roberts et al. 2001, Sydenstricker et al. 2005) have shown that house finches experimentally infected with the same inoculum dose can develop different courses of disease. A small percentage of finches tend to develop severe, protracted disease. Another goal of this work is to examine if pathogen load and antibody profiles (serum and tear antibodies) of finches that show "acute" disease differ from finches that show "chronic" disease.

Prior to this work, little was known about the house finch antibody response to MG, since there is a lack of commercially available products to facilitate this research. Very few reagents have been developed to detect immunoglobulins of passerine birds, and monoclonal antibodies developed against chicken immunoglobulins often show poor cross-reactivity with those of other avian species, especially to those distantly related to the chicken (Jeurissen and Janse 1998, for review see Schultz and Magor 2008). This work describes the development of a polyclonal reagent that is specific for house finch IgA and uses the reagent to characterize the local (lachrymal) antibody response to MG (Chapters 5, 6, 7).

It is speculated that MG varies cell surface antigens in order to escape the host antibody response. It has been demonstrated both *in vitro* and *in vivo* (in the chicken) that MG has the ability to vary cell surface proteins, most notably, the VlhA lipoprotein. A large multi-gene family is responsible for encoding the protein, and one gene is expressed at a time. Implicated functions of the VlhA protein include adherence, hemagglutination, and escaping the host antibodies (Citti et al. 2005). The ability to vary surface proteins is not unique to MG. *M.*

synoviae and *M. imitans*, pathogens of poultry, also have multi-gene families thought to be homologous to the MG VlhA. There is genetic sequence similarity between the *vlhA* genes of MG and *M. synoviae*; however, in *M. synoviae*, only a single copy of the complete *vlhA* gene exists in the genome and all others are pseudogenes and in MG, all or most genes are translationally competent (Bencina 2002).

VlhA protein expression differs among poultry isolates of MG (Glew et al. 1995). Another objective of this thesis is to determine if circulating house finch MG strains also differ in VlhA expression. Additionally, *in vivo* variation in VlhA expression is studied in house finches experimentally inoculated with MG, with particular emphasis on birds that show chronic or severe disease.

Chapter 2 provides a literature review on relevant topics to this thesis, including an overview of the genus *Mycoplasma*, mycoplasmosis in poultry and other birds, chicken immune response to MG, VlhA variation, and the role of the avian Harderian gland in immune defense of the eye. Thesis goals are listed at the end of chapter 2, and chapter 3 provides an overview of the general experimental design used for this thesis.

CHAPTER 2

Literature review

2.1. Pathogen and host

2.1.1. The genus Mycoplasma

Members of the genus *Mycoplasma* belong to the Class *Mollicutes* (*molis* – "soft"; *cutis* – "skin"). Mycoplasma species lack a cell wall and have small genomes. Also unique to *Mycoplasma* and some other members of the *Mollicutes* is the presence of cholesterol in the plasma membrane (Razin 1998). Mycoplasma species are parasites of plants, arthropods, fish, reptiles, birds, humans, and other mammals. Though they are widespread pathogens, they often have strict host and tissue specificity. As a whole, these organisms are often difficult to culture, likely due to their dependence on many exogenous nutrients or the presence of unknown inhibitory substances in cultures (Razin 1998).

Mycoplasmas typically infect mucosal surfaces such as the respiratory and urogenital tracts, eyes, mammary gland, and joints. Three of the most common human pathogens include *M. pneumoniae*, a respiratory pathogen, and *M. hominis* and *M. genitalium*, primarily pathogens of the urogenital tract (Waites and Talkington 2005).

There are numerous *Mycoplasma* species that infect animals. *Mycoplasma* species are important respiratory pathogens of cattle, causing pneumonia (*M. bovis*) and contagious bovine pleuropneumonia (*M. mycoides mycoides* small colony type). *M. capricolum capripneumoniae* is the cause of contagious caprine pleuropneumonia, a highly fatal disease of goats in Europe, Africa, and Asia. There are several other species of *Mycoplasma* causing pneumonia in small ruminants (*M. mycoides capri* and *M. mycoides mycoides* large colony type) and pigs (*M. hyopneumoniae*). In rats, *M. pulmonis* is an agent that contributes to chronic respiratory disease,

a condition that is often poorly responsive to antibiotic therapy (Donnelly 2004). There are also several hemotropic mycoplasmas infecting veterinary species that can cause anemia (e.g., dogs – *M. haemocanis*, cats – *M. haemofelis*, pigs – *M. suis*, cattle – *M. we*nyonii, sheep/goats – *M. ovis*) (Kahn 2010). The avian mycoplasmas will be discussed in the following sections.

2.1.2. Mycoplasmosis in birds

Approximately 23 species of *Mycoplasma* are known to infect birds. Most of these species have been found in poultry, although 17 are also found in free-living birds, and 5 species have only been identified in free-living avian species (Luttrell and Fischer 2007).

Mycoplasmosis in poultry has been well studied due to the significant agricultural and economical impacts of the diseases. Four species are commonly found as poultry pathogens, including *M. gallisepticum*, *M. synoviae*, *M. meleagridis*, and *M. iowae*. *M. gallisepticum* is the most economically important and is discussed in detail in section 2.1.3. *M. synoviae* typically infects chickens and turkeys and causes synovitis or respiratory disease. *M. meleagridis* causes respiratory disease, skeletal abnormalities, and decreased growth in turkeys. *M. iowae* is a cause of embryonic mortality in turkeys (Kleven 1998a, Kleven 1998b).

M. gallisepticum and *M. sturni* cause conjunctivitis in North American passerines; *M. gallisepticum* is most common in finches and *M. sturni* has been identified in European starlings (*Sturnus vulgaris*), Northern mockingbirds (*Mimus polyglottos*), blue jays (*Cyanocitta cristata*), American crows (*Corvus brachyrhynchos*), and American robins (*Turdus migratorius*) (Forsyth et al. 1996, Ley et al. 1998, Wellehan et al. 2001). Three species of *Mycoplasma* have also been isolated from European raptors with respiratory disease, including *M. buteonis* from common buzzards (*Buteo buteo)*, *M. falconis* from saker falcons (*Falco cherrug*) and *M. gypis* from

griffon vultures (*Gyps fulvus*) (Poveda et al. 1994). Other species of *Mycoplasma* have been isolated from free-living birds, including turkeys, ducks, and pigeons (Luttell and Fischer 2007).

2.1.3. Mycoplasma gallisepticum infection in poultry

M. gallisepticum (MG) is a cause of chronic respiratory disease in chickens and turkeys characterized by coughing, nasal discharge, and conjunctivitis (Ley 2003). It can also cause severe airsacculitis, especially in conjunction with other bacterial infections (such as *Escherichia coli*) and viral infections (such as infectious bronchitis or Newcastle disease). Weight loss and decreased egg production are common during infection. Salpingitis and encephalitis can also occur, though less commonly. Serologically positive flocks may also show few, if any, clinical signs. Turkeys tend to develop more severe clinical signs than chickens, including sinusitis, respiratory distress, depression, and weight loss. Infection can last for months in flocks if untreated. Disease tends to be more severe and protracted in colder months and in younger birds (Ley 2003).

On necropsy, gross lesions often include exudate in the nasal passages, trachea, bronchi, and air sacs, and sometimes pneumonia. Microscopic lesions include thickening of mucous membranes associated with infiltration of mononuclear cells (often predominantly lymphocytes and plasma cells) and hyperplasia of glands. Destruction of cilia can be observed in the trachea (Ley 2003). MG antigen can be detected by immunohistochemistry in the trachea, conjunctiva, nasal turbinates, and air sacs of experimentally inoculated chickens (Gharaibeh and Hailat 2011).

MG transmission occurs via aerosol or droplets associated with direct or indirect contact and via fomites (such as dust, feathers, and farm personnel). Vertical transmission can also

occur, especially when infected individuals are laying eggs during the acute phase of the disease (Ley 2003, Lin and Kleven 1982).

It is thought that MG resides on the surface of the respiratory tract and conjunctiva, although systemic infection can occur, resulting in spread to other organs such as the brain (e.g., Chin et al. 1991). *In vitro* and *in vivo* studies suggest that MG can adhere to and invade nonphagocytic cells (Winner et al. 2000, Vogl et al. 2008), which may encourage systemic spread and also allow the organism to evade the host immune system via an intracellular route.

Management of MG in poultry flocks involves establishment of MG-free breeder flocks, use of vaccines, and use of antimicrobial drugs. Both bacterins and live vaccines have been used in the past. Currently, a modified live 6/85 strain of MG is commercially available (Intervet, Millsboro, DE), and is administered via aerosol route (spray). Another commercially available vaccine is a modified live ts-11 strain (Bioproperties, Ringwood, Australia), and is administered via eye-drop route. MG is sensitive to macrolides, tetracyclines, and fluoroquinolones. More commonly used flock treatments include oxytetracycline or chlortetracycline in the feed and tylosin administered in the drinking water (Ley 2003).

2.1.4. Overview of Mycoplasma gallisepticum in house finches

Historically, house finches (*Haemorhous,* formerly *Carpodacus*, *mexicanus*) were distributed along the western half of North America. A number of captive house finches were introduced to Long Island, NY in the 1940s, and from that time, the population spread successfully throughout the east coast. House finches commonly take advantage of bird feeders and have a preference for human-modified landscapes (Elliot and Arbib 1953, Hill 1993)

In early 1994, *Mycoplasma gallisepticum* (MG) emerged in free-living house finches in Maryland and Virginia and subsequently spread throughout the eastern parts of the United States, causing major declines throughout the introduced population of the species (Hochachka and Dhondt 2000). In 2002, the disease was documented in Montana, part of the native range of the species, and reached Oregon by 2004 (Ley et al. 2006). In the native western population, the epidemic has progressed more slowly, with lower prevalence levels than in eastern populations (Dhondt et al. 2006). It is thought that the disease is transmitted via direct contact (Ley 2003) and by fomites (e.g., birdfeeders) (Dhondt et al. 2007a).

The clinical signs of infection in house finches usually include unilateral or bilateral conjunctivitis. In house finches inoculated ocularly with MG, signs of conjunctivitis, sometimes in addition to rhinitis, begin between two and six days post-infection. All infected birds remain MG-positive by PCR for up to three weeks post-infection, and some remain PCR positive with clinical signs for greater than twenty-one weeks (Kollias et al. 2004). Interestingly, Davis (2010) documented a left-bias pattern in free-living house finches with conjunctivitis, that is, left eyes tend to have more severe conjunctivitis than right eyes. This pattern was speculated to be due to an inherent "handedness" in the finches, or preference to turn their head to a specific (left) side, leading to increased fomite exposure on that side of the head.

2.1.5. M. gallisepticum infection in other bird species

Experimentally infected house sparrows (*Passer domesticus*) show either no clinical signs or short-lived mild conjunctivitis (Dhondt et al. 2008). Eight out of ten experimentally infected American goldfinches (*Carduelis tristis)* developed clinical signs. Goldfinches may serve as

asymptomatic carriers for MG since they tend to clear clinical signs but remain infectious (Dhondt et al. 2008, Dhondt et al. 2013).

Farmer et al. (2005) found that clinical signs were not apparent in house sparrows, chipping sparrows (*Spizella passerine*), zebra finches (*Taeniopygia guttata*) and budgerigars (*Melopsittacus undulates*) after infection with MG. Though they could not detect clinical signs, choanal samples of the birds taken after infection were positive for MG in all but the chipping sparrows.

2.1.6. Variations in clinical disease

Although challenged with identical doses of MG, experimentally infected house finches exhibit variations in disease progression, such as onset of conjunctivitis, the severity of conjunctivitis, and duration of clinical disease (Kollias et al. 2004, Sydenstricker et al. 2005). Roberts et al. (2001) found that 73% of infected house finches developed acute, self-limiting conjunctivitis while the remaining 27% suffered from chronic disease and had a higher mortality rate. The reasons chronic disease only occurs in a fraction of individuals are unclear.

2.1.7. Methods of detecting M. gallisepticum infection in house finches prior to my research

In house finches, PCR was used to detect MG in conjunctival swab samples. The conventional PCR assay was designed for detection of MG in poultry samples using the 16S rRNA gene of MG (Garcia et al. 2005, Lauerman 1998). A commercially available serum plate agglutination test (Intervet, Millsboro, DE) was used to screen for presence of MG-specific antibodies in the serum (Kleven 1998b). Eye lesions are scored for a measure of disease state. This is a visual measure based on a score from 0 to 3, with 0 being a normal eye and 3 representing discoloration

of the conjunctiva, severe edema of the conjunctiva, supraorbital area, and infraorbital sinus, epiphora and feather matting (Sydenstricker et al. 2005).

2.2. Immunology

2.2.1. Immunological responses during Mycoplasma infection

Mycoplasma species are known for their ability to cause chronic disease while persisting in their hosts in spite of very active immune responses directed against them. Apparently ineffective immune responses lead to the immunopathologic lesions often observed during mycoplasmal infections (e.g., Cartner et al. 1998, see Simecka 2005 for review). Even after recovery from disease, chickens may remain as carriers of MG for many months (Bencina and Dorrer 1984).

Early studies on the immune response of chickens to MG suggest that resistance is mediated by immunoglobulins, since bursectomized chickens show increased susceptibility to infection (Alder et al. 1973, Lam and Lin 1984). Earlier studies also noted that there is little correlation between serum immunoglobulin levels and immunity (e.g., Talkington and Kleven 1985) and various studies have provided evidence that local immunity plays an important role during MG infection. Yagihashi and Tajima (1986) found that antibody titers in tracheal washings of infected chickens increased during the three weeks post-infection and this increase was correlated with decreasing tracheal inflammatory lesions. Re-exposed chickens recovered faster and had less severe lesions. Chhabra and Goel (1980) found that regression of lesions was correlated with peak antibody response in sera and tracheal washings. Avakian and Kleven (1990) found that chickens infected with MG responded with antibodies to over twenty proteins, determined by western blot. Avakian and Ley (1993) further demonstrated the role of local

immune response by using *in vitro* assays to show that tracheal washings from infected chickens inhibited MG growth.

More recently, in an experiment by Javed et al. (2005), chickens were either vaccinated or sham-vaccinated, and subsequently challenged with pathogenic MG strain R_{low}. After challenge, the vaccinated birds had more MG-specific IgG and IgA-secreting plasma B cells within the trachea and developed only mild tracheal lesions, while the sham-vaccinated birds had larger numbers of B, CD4+, and CD8+ cells infiltrating the trachea. Gaunson et al. (2006) characterized the infiltration of lymphocytes into the chicken trachea at multiple time points after MG infection. In unvaccinated birds, CD8+TCR+ and CD4+ cells increased in the trachea after infection. In the early stages of disease, a majority of CD8+ cells were TCR-, suggestive of natural killer (NK) cells. While the study found T lymphocytes are involved in response to MG, recovery from infection in naïve birds correlated well with the development of lymphoid follicles that were composed of mostly B-cells (3-6 weeks post-infection), further underscoring the importance of local antibody responses.

2.2.2. Immune responses of house finches to M. gallisepticum

The route of infection in free-living house finches is not known for certain, but experimental infection by inoculation into the conjunctiva results in clinical signs seen in free-living birds (e.g., see Luttrell et al. 1996 for disease in free-living finches; Sydenstricker et al. 2006 for disease in finches after experimental inoculation). In addition, the organism can be cultured from conjunctival swabs of free-living birds (Hartup et al. 2000, Ley et al. 1996).

Dhondt et al. (2007b) found that both conjunctival and oral inoculation results in seroconversion, as measured by serum plate agglutination, however, only two out of ten orally

inoculated house finches showed signs of conjunctivitis, whereas all birds exhibited conjunctivitis when inoculated into the conjunctiva. It is also been demonstrated that birds develop partial immunity after initial infection; re-exposed birds are susceptible to infection and develop disease after secondary exposure, however, they developed milder disease for a shorter duration of time (Sydenstricker et al. 2005).

Using microsatellite DNA analysis to examine genetic heterozygosity, Hawley et al. (2005) found that house finches with greater heterozygosity developed less severe disease after conjunctival MG inoculation. Heterozygosity was also positively correlated with cell-mediated immunity as measured by T-cell proliferative response to phytohaemagglutinin, but was not correlated with humoral response to sheep red blood cells. The authors suggested that differences in the cell-mediated immune response may explain why greater heterozygosity is associated with resistance to MG. Additionally, Hawley and Fleischer (2012) found that house finches with intermediate to high levels of MHC Class II diversity showed the lowest disease severity after experimental infection with MG, compared with finches that had low MHC Class II diversity. MHC diversity may be particularly important during MG infection since MG alters expression of cell surface antigen (discussed in 2.2.4).

Bonneaud et al. (2011) experimentally infected house finches from two North American populations: one from an eastern population which was exposed to MG for 12 years (Alabama) and a western population without prior exposure to MG (Arizona). They found that finches from the exposed population had 33% lower MG loads in their conjunctivae two weeks post-infection, as measured by the *mgc2* quantitative PCR assay described in chapter 4. Using a cDNA microarray, they also found that finches from the eastern population up-regulated more immune-related genes, providing evidence for evolution of resistance in the eastern population.

Microscopic lesions of house finches with MG infection further confirm the involvement of an active immune response. Luttrell et al. (1996) characterized microscopic lesions in a number of free-living house finches with MG infection. Most had moderate to severe hyperplasia of epithelial and lymphoid tissues of the conjunctiva, with many plasma cells and some lymphocytes and macrophages infiltrating the submucosa. A few birds had keratitis characterized by mild to moderate infiltration of plasma cells, lymphocytes, and heterophils in the cornea, along with hyperplasia of corneal epithelium. In a study of captive house finches with MG infection, Luttrell et al. (1998) found similar lesions in the conjunctiva and cornea, in addition to nasal turbinate lesions which included submucosal lymphocyte and plasma cell infiltrates and focal mucosal necrosis. In the trachea, mucosal and submucosal thickening from epithelial hyperplasia and lymphoplasmacytic infiltrate were also present.

2.2.3. Local defense of the avian eye: the role of the Harderian gland

As discussed, existing evidence suggests that the local antibody response has an important role in controlling MG infection in chickens. Since MG infects ocular tissue in finches, the local response likely involves peripheral lymphoid tissue associated with the eye. In avian species, the principal peripheral lymphoid tissue associated with the eye resides in the Harderian gland. Harderian glands are found in terrestrial vertebrates that have a nictitating membrane (Shirama et al. 1996). It is thought that its secretions generally act to lubricate the eye, although other functions have been proposed and are most often species-dependent. In birds, there are large numbers of antibody producing plasma B cells within the Harderian gland (Bang and Bang 1968). While the gland contains predominately B cells, a small number of helper T cells can also be present (Albini et al. 1974).

Mueller et al. (1971) established the role of the Harderian gland in the local defense of the eye by demonstrating that when an antigen is administered intravenously, antibody production did not take place in the gland. However, when antigen was inoculated ocularly, the Harderian gland produced antigen-specific antibodies. Baba et al. (1988) found that surgical removal of the Harderian gland in day-old chicks resulted in greatly decreased immunoglobulin concentrations in the lachrymal fluid. Lachrymal IgA concentration appeared to be especially dependent on the presence of the gland.

Karaca and Lam (1989) found that the Harderian gland is not necessary for resistance to MG infection in chickens. They surgically removed the gland in chicks prior to administering an intranasal vaccine for MG and subsequently measured antibody response in the sera and in tracheal washings. They reported that after vaccination, chicks without the gland still produced an antibody response, in sera and tracheal washings, to the challenge strain. However, given the background discussed above, the Harderian gland likely protects against antigens encountered in the eye, and so it is not surprising that the gland may not be necessary to produce specific antibodies for antigens inoculated via other routes. Also, the antibody-rich secretions of the Harderian gland drain into the conjunctiva at the base of the nictitating membrane (Shirama et al. 1996), and therefore it is generally assumed that the tears contain the highest concentration of Harderian gland-derived antibodies, not the tracheal washings.

Thus, the avian Harderian gland is important for defense against antigens inoculated onto the eye and conjunctival tissues. As in mammals, IgA is the predominant antibody involved in protection of the mucosal surface (Tizard 2002). Since the Harderian gland appears to be the primary source of IgA in the lachrymal fluid (Baba et al. 1988), it may serve as a vital player in local antibody response against MG in the house finch conjunctiva.

2.2.4. Mycoplasma lipoproteins and the role of MG's vlhA gene family in immune evasion

Mycoplasma species are known for their ability to cause chronic infection in their hosts. Antigenic variation via cell surface lipoproteins may aid in the ability to cause prolonged infection. In these species, proteins make up over two-thirds of plasma membrane mass, with membrane lipids composing the rest. Unlike other eubacteria, membrane lipoproteins are abundant in the membrane (Razin 1999). Membrane lipoproteins appear to be the major antigens in *Mycoplasma* species, and many cell surface antigens have been found to undergo antigenic or size variation (Razin 1999). In these species, all membrane-bound lipoproteins contain a lipoylated amino-terminal cysteinyl residue, and these lipoproteins comprise 4-10% of the coding sequences in the genome (Rocha et al. 2005). Surface lipoproteins are often the target of the humoral immune response (Rocha et al. 2005). *Mycoplasma* species undergo variation of surface antigens *in vitro* (e.g., Zhang and Wise 2001) and *in vivo* (Levisohn et al. 1995), likely an adaptation to evade the host antibody response.

The MG *vlhA* gene family, formerly referred to as the pMGA gene family, encodes variants of VlhA protein, a major surface lipoprotein. It is thought that the VlhA protein plays a role in adherence, hemagglutination, and evasion of host antibodies (Citti et al. 2005).

Markham and colleagues at the University of Melbourne have contributed a great deal to our knowledge of the MG VlhA protein. Markham et al. (1992) found that hemagglutination of chicken erythrocytes by MG strain S6 was inhibited when MG S6 was incubated with pMGA (VlhA)-specific monoclonal antibodies, providing evidence of its possible role in attachment to host cells. The monoclonal antibodies were also used to identify a protein band with a M_r of 67,000, and electron microscopy was used to show that the VlhA protein was located on the cell

surface of MG. Markham et al. also sequenced the amino terminus of pMGA, which did not have significant homology to any other protein in major databases.

Markham et al. (1993) purified a protein using a monoclonal antibody against pMGA, and subsequently sequenced two proteins (pMGA1.2 and 1.3) that were similar but not identical to the pMGA protein described previously (pMGA1.1), providing evidence for the existence of a gene family encoding these homologous proteins. Based on the sequence of pMGA1.2, it appeared that the protein lacked a significant internal hydrophobic region, but had a protein sequence flanking the amino-terminal cysteine that could form a signal peptidase II cleavage site. Such a site could lead to addition of a fatty acyl group to its alpha-amino group providing an amino terminal anchor for the protein. Markham et al. (1994) sequenced several pMGA genes and found that all had GAA repeats at the 5' region of the gene, ranging from 10-15 repeats, that were located upstream from the start codon of each gene. Only a single *vlhA* gene is expressed at a time and expression requires a trinucleotide motif ($[GAA]_{12}$) 5' to its promoter (Glew et al., 1998). Change in VlhA expression likely occurs due to slipped-strand mispairing at the trinucleotide repeat during DNA synthesis (Citti et al. 2005).

The complete genome of the strain R_{low} has been sequenced, and it has been found that the *vlhA* gene family comprises approximately ten percent of the MG genome. In MG strain R_{low}, there are 43 *vlhA* genes that are present at five loci. Sequence identity among the gene variants ranges from 41-99% (Papazisi et al. 2003). Among strains of MG, *vlhA* gene number ranges from 32 to 70 (Baseggio et al. 1996), determined by Southern blotting using a probe that was complimentary to a leader sequence present in *vlhA* genes. Genome comparison of strain R_{low} (a virulent low-passage strain) and strain F (an attenuated strain) shows great variability in the *vlhA*

gene family, and provides evidence for genomic inversion events, gene duplications, and insertion/deletion events in this gene family (Szczepanek et al. 2010).

The MG VlhA surface protein undergoes phenotypic variation *in vitro* in response to VlhA-specific antibodies. To study phenotypic variation, Markham et al. (1998) utilized MG strain S6, a monoclonal antibody directed against an epitope on pMGA1.1, and polyclonal antisera against pMGA antigens. When included in the growth media, both the monoclonal antibody and polyclonal antisera resulted in cessation of pMGA1.1 expression; instead, the cultured cells expressed a different member of the pMGA gene family. Additionally, when these cells were then allowed to grow in the absence of the antibody, progeny of these cells regained expression of the pMGA antigen (Markham et al. 1998).

In vivo variation of the VlhA protein has also been documented during MG infection in chickens (Glew et al. 2000). While it is thought that VlhA phenotypic variation is a possible mechanism for host antibody evasion, VlhA switching also occurs in the absence of detectable antibodies. Glew et al. (2000) studied MG VlhA switching in chickens by examining colonies of MG grown from post-inoculation tracheal wash samples. They found that by two days post-aerosol inoculation, 40% of MG cells ceased expression of the VlhA surface protein expressed at the time of inoculation; however, VlhA specific antibodies were not detected in serum and tracheal samples until day 6 post-inoculation. Thus, it may be possible that the *vlhA* gene family plays a role in other functions beside antibody evasion, such as colonization (Glew et al. 2000).

One possible mechanism for VlhA switching is that antibodies select for individual cells within a population that have switched off expression of the target VlhA protein by chance. Another possibility is that binding of the antibody to the MG cell influences the rate of VlhA

phase variation (Citti et al. 2005). The work by Glew et al. described above, however, suggests that VlhA variation can also occur via an antibody independent mechanism.

Very recent work by Tulman et al. (2012) compared the genomes of eight MG isolates collected from free-living house finches between 1994-2008 from different geographic locations. The authors found that much of the house finch MG genome is highly conserved between the isolates, with a major exception being the *vlhA* genes, which vary in presence and genomic location. House finch MG genomes contain 33 to 48 *vlhA* genes, and many genes have homologues at similar loci in poultry strains R and F, although some have no homologues.

2.3. Thesis goals

The goal of my thesis was to characterize house finch local and systemic antibody responses to *Mycoplasma gallisepticum* and address several questions.

1) Temporal and spatial genotypic variation in house finch MG has been documented (Pillai et al. 2003, Tulman et al. 2012), but phenotypic variation in pathogenicity and immunogenicity has not been examined. Do circulating house finch strains or strain variants of MG differ in virulence and immunogenicity?
2) Previous studies (Kollias et al. 2004, Roberts et al. 2001, Sydenstricker et al. 2005) have shown that house finches experimentally infected with the same inoculum dose can develop very different courses of disease. Do antibody profiles (serum and tear antibodies) of birds that show "acute" disease differ from birds that show "chronic" disease? Is there a difference in pathogen load between these two groups?
3) VlhA protein expression differs among poultry isolates of MG (Glew et al. 1995). Do circulating house finch MG strains also differ in VlhA expression?

4) Does VlhA expression vary *in vivo* in house finches experimentally infected with MG? What role might this play in chronic infections?

2.4. Significance of study

2.4.1. Broadening the knowledge of the avian immune system

The chicken has provided us with the majority of our knowledge of the avian immune system. Knowledge of immunity (especially mucosal immunity) of non-galliform birds is greatly limited. It will be necessary to study other avian species to substantiate the generalizations we have made about avian immunity. In order to fully study the immune system of non-galliform species, new reagents must be developed that can detect various components of the immune system. More specifically, data on immunity to MG in wild birds was limited, with plate agglutination being the predominant serologic test utilized prior to this work (Luttrell and Fischer 2007). The development of an ELISA for use in house finches allowed for quantification of antibody response and likely greater specificity than plate agglutination.

Broadening knowledge of "comparative mycoplasmology"

Although each *Mycoplasma* species has a narrow host range, mycoplasmas infect a wide diversity of hosts, including fish, reptiles, birds, and mammals. *Mycoplasma* species cause a multitude of diseases in humans (Waites and Talkington 2005) and domesticated animals (Markham and Noormohammadi 2005) as discussed in previous sections. "Comparative mycoplasmology" is pertinent to human and domestic animal disease since it will broaden our understanding of mycoplasma-host interactions and pathogenesis, and subsequently allow for further exploration of strategies to control infection.

Conservation

While house finches appear to be the major free-living host species affected by MG, an increasing number of species are being documented as PCR- and/or antibody-positive for MG. Conjunctivitis and positive serum plate agglutination tests have less commonly been documented in other songbirds, such as American goldfinches and purple finches (Hartup et al. 2000, Hartup et al. 2001). Evidence of infection has been identified in over 30 other species, including mourning dove (*Zenaida macroura)*, evening grosbeak (*Coccothraustes vespertinus),* pine grosbeak (*Pinicola enucleator)*, wood thrush (*Hylocichla mustelina)*, and northern cardinal (*Cardinalis cardinalis)* (J. DeCoste, personal comm.). In the eastern range of the house finch, the MG epidemic has caused a major decline in house finch abundance (Hochachka and Dhondt 2000). If MG is capable of infecting other species, it is possible MG infection could cause population declines in more vulnerable species in the future. The greater knowledge we have of this disease, the better our understanding and ability to assess threats to other species in the future.

CHAPTER 3

General experimental design

To investigate virulence and immunogenicity of *M. gallisepticum* (MG) and address the thesis goals listed in chapter 2, experimental infections were carried out using several isolates of MG. These isolates were collected from house finches from various geographic locations and time points. Before these experiments were initiated, diagnostic tests were designed for application in this system. To detect pathogen, a quantitative PCR was developed for detection of MG in conjunctival swab samples (Chapter 4). This assay utilizes the *mgc2* gene, present as one copy per bacterial cell. A quantitative PCR was also developed for use as a host (house finch) cell control, and was based on the *rag-1* gene, present in the house finch cell as two copies per cell.

House finch antibody response to MG was investigated in this thesis. Unfortunately, there is a general paucity of immunological reagents for non-agricultural avian species. The development of class-specific monoclonal antibodies to chicken immunoglobulins has greatly facilitated past research. Many of these monoclonal antibodies developed against chicken immunoglobulins, however, show poor cross-reactivity with those of other avian species, especially to those distantly related to the chicken (Jeurissen and Janse 1998, for review see Schultz and Magor 2008).

Few reagents have been developed to detect immunoglobulins of passerine birds. Commercial polyclonal antibodies are available for the detection of bird IgM (Immunology Consultants Laboratory, Newberg, OR) and IgY (Bethyl Laboratories, Montgomery, TX). These polyclonal antibodies were generated by immunizing rabbits and goats with immunoglobulin

from at least one species of passerine bird (e.g., robin and crow IgM and sparrow IgY). To date, no reagents have been developed against IgA from a passerine bird.

To investigate local immunity, a reagent that recognizes house finch IgA was developed (Chapter 5). A fragment of the house finch IgA heavy chain was PCR-amplified from house finch spleen cDNA, sequenced, expressed using a bacterial expression system. This protein was then used to generate house finch IgA-specific rabbit antisera. A commercially available ELISA was then modified for use in house finches for measurement of MG-specific lachrymal IgA and serum IgG(Y).

After the development of the quantitative PCR and ELISAs, MG virulence and house finch antibody responses were studied in several experimental infections (Chapter 6). In experiment 1, house finches were inoculated via conjunctivae with MG isolates collected from house finches in Virginia (1994) and California (2006). In experiment 2, house finches were inoculated with the Virginia 1994 isolate and an isolate collected from a house finch in North Carolina (2006). Eye lesion scores, MG load and house finch antibody response were measured in both experiments.

In Chapter 7, chronic disease was studied with experimental inoculations of house finches using VA1994 and North Carolina (NC1995, NC1996, NC2006, NC2008) field isolates. Eye lesion scores, MG load and antibody responses were once again measured. Using a monoclonal antibody specific for a variant of MG VlhA immunodominant surface protein, *in vivo* VlhA changes during infection in the house finch were documented.

CHAPTER 4

Detection and quantification of *Mycoplasma gallisepticum* genome load in conjunctival samples of experimentally infected house finches (*Carpodacus mexicanus*) using real-time polymerase chain reaction*

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4.1 Abstract

A TaqMan®-based real-time, quantitative polymerase chain reaction (qPCR) assay utilizing the *mgc2* gene was developed to detect *Mycoplasma gallisepticum* (MG) in conjunctival swabs of experimentally infected house finches. The assay was demonstrated to be quantitative by the standard curve method with reproducible results within runs and between runs. The detection limit of the *mgc2* assay was examined using two standards. The test had a detection limit of less than 14 copies per reaction when tested with a plasmid standard and less than 10 copies per reaction when tested with *M. gallisepticum* genomic DNA. All *M. gallisepticum*-negative birds (10 specific pathogen free chickens and 10 house finches) were negative by *mgc2* qPCR assay. Existing evidence suggests that an important part of *M. gallisepticum* pathogenesis includes both its attachment to and invasion of host cells. Thus, our test also made use of *rag-1* as an internal control gene. The *rag-1* qPCR results showed that host cell quantity varied greatly between conjunctival samples. After inoculation, *M. gallisepticum* levels in the house finch conjunctiva increased over the 7-day period post infection. The bird with the most pronounced clinical conjunctivitis harboured the highest level of *M. gallisepticum* and the bird that did not develop conjunctivitis had very low numbers of *M. gallisepticum*. Thus, it appears that development of conjunctivitis may correlate with *M.gallisepticum* load.

4.2 Introduction

Mycoplasma gallisepticum is a prokaryotic pathogen that causes chronic respiratory disease in chickens and infectious sinusitis in turkeys. Disease resulting from *M. gallisepticum* infection has considerable economic consequences for the poultry industry worldwide. In 1994, *M. gallisepticum* was detected in house finches (*Carpodacus mexicanus*) in the eastern part of North America (Ley et al. 1996). The infection caused major declines throughout the eastern population and eventually spread to the native western range of the house finch (Ley et al. 2006). The most prominent clinical sign of infection in finches is unilateral or bilateral conjunctivitis, which in experimentally infected birds develops approximately 4 days post infection (p.i.) (Kollias et al. 2004).

Several real-time quantitative polymerase chain reaction (qPCR) assays have been developed to measure *M. gallisepticum* in tracheal and palatine cleft swabs of domestic chickens. Two SYBR Green-based assays that use standard curves based on colony-forming units (CFUs) have been described. One assay utilizes the 16S rRNA gene (Mekkes and Feberwee 2005), and the other utilizes the *mga_0319* lipoprotein gene (Carli and Eyigor 2003). Problems associated with these assays have been a lack of sensitivity or cross-reaction with other *Mycoplasma* species. Callison et al. (2006) developed a TaqMan®-based assay that quantified *M. gallisepticum* based on *mga_0319* gene copy numbers using a plasmid standard, instead of CFUs.

Lacking in the previously developed qPCR assays is the use of a host housekeeping gene. The measurement of a host gene is an important control to account for differences in swabbing methods, DNA extraction efficiencies, and inflammatory responses. There is also increasing evidence for the close association of *M. gallisepticum* with host cells, including both adhesion to

and invasion of host cells. In other *Mycoplasma* species, adhesion to host cells plays a vital role in pathogenesis (Balish and Krause 2005). This is also true for *M. gallisepticum*, as genes and corresponding proteins involved in cytadhesion have been identified and characterized (Keeler et al. 1996, Goh et al. 1998, Hnatow et al. 1998, Papazisi et al. 2002). There is also evidence that *M. gallisepticum* has the ability to invade host cells. Winner et al. (2000) demonstrated that *M. gallisepticum* is internalized by human epithelial cells and chicken embryo fibroblasts *in vitro*. More recently, studies by Vogl et al. (2008) provided evidence that *M. gallisepticum* can invade and adhere to chicken erythrocytes *in vitro* and can invade erythrocytes *in vivo* after aerosol inoculation.

In many polymerase chain reaction (PCR) assays, the 18S rRNA gene has been used as an endogenous control for eukaryotic DNA (for example, Bai et al. 2004, Casabianca et al. 2004) and RNA (Li et al. 2005). However, the use of this gene as a DNA control has several disadvantages. The gene is present in multiple copies within the eukaryotic genome and copy numbers vary among species and at the individual level (Long and Dawid 1980). For example, the 18S rRNA copy number varies between chicken strains and among individuals within a given strain. Delany (2000) found copy numbers ranging from 302 to 422 in broiler lines. Moreover, the 18S rRNA copy number in the house finch is unknown; therefore, quantification of host cell number is not feasible using this gene.

Conventional PCR has been used to identify infected house finches using conjunctival swabs. The *mgc2* gene, which encodes a cytadhesin protein (Hnatow et al. 1998), is currently the one of the preferred gene targets for this assay, due to its specificity for *M. gallisepticum* (Garcia et al. 2005). We selected this gene for the development of our qPCR to quantify the level of *M. gallisepticum* infection in house finches. The recombination-activating gene 1 (*rag-1*), which is

present in two copies/diploid cell in the genome of higher vertebrates (Groth and Barrowclough 1999), was selected to quantify the number of host cells in conjunctival swab samples.

4.3 Materials and Methods

Sample sources

Serum samples and conjunctival samples from both eyes were obtained from 10 chickens from the specific pathogen free P2a (Weinstock and Schat 1987) flock at Cornell University. Conjunctival samples were collected using cotton-tipped wooden-handle swabs (product #14-960-3N; Fisher Scientific, Pittsburgh, Pennsylvania, USA), placed in 300 µl tryptose phosphate broth and kept at 4°C until the swab was discarded. Then the remaining sample was stored at −28°C until DNA extraction. Blood was collected from the wing vein on the date of swab collection and also 4 weeks later to confirm *M. gallisepticum*-negative antibody status using serum plate agglutination assays (Charles River Laboratories, Wilmington, Massachusetts, USA). The P2a line has been negative for *M. gallisepticum* antibodies for at least 20 years and is tested monthly to confirm its negative status.

One-year-hatchling house finches were trapped in Tompkins County, New York, USA (42°51′N, 76°34′W) during October and November 2006 using mist nets under permits (LCP 99-039) from the New York State Department of Environmental Conservation (Albany, New York, USA) and a federal collecting permit (PRT 802829). Birds were placed in quarantine for a minimum of 2 weeks, and blood was collected from the wing vein into lithium-heparinized microcapillary tubes and screened for the presence of *M. gallisepticum*-specific antibody using the serum plate agglutination test (Intervet, Millsboro, Delaware, USA) (Kleven 1998). The house finches were kept in individual wire bar cages with metal barriers between them to prevent

mechanical spread of *M. gallisepticum*. Water and a pelleted diet (Roudybush, Inc., Cameron Park, California, USA) were provided *ad libitum*.

Ten additional 1-year-hatchling house finches were sampled during October 2007. After *M. gallisepticum* antibody-negative status was confirmed with serum plate agglutination, conjunctival swabs were obtained to serve as *M. gallisepticum*-negative samples. Samples from the left and right eyes were pooled for each bird.

Experimental infection

Five birds were inoculated in the conjunctival sac of the right and left eyes with 50 µl *M. gallisepticum* inoculum (3.24 x 10^5 CFU/ml) at day 0. Conjunctival swabs were collected at day 0 (pre-inoculation), and 1, 3, 5, and 7 days p.i. Conjunctival samples were collected using cotton-tipped swabs, placed in 300 µl tryptose phosphate broth, and kept at 4°C until the swab was removed. The remaining sample was then stored at −28°C until DNA extraction. Samples for the right and left eyes were processed separately so that the *M. gallisepticum* load for each eye could be determined. Physical signs of *M. gallisepticum* infection were quantified by scoring the severity of the inflammatory process in each eye using a 0 to 3 scale as described by Sydenstricker et al. (2006): 0=no visible inflammation; 1=pink conjunctival discolouration and slight periorbital oedema, 2=pink conjunctival discolouration, slight to moderate periorbital oedema, and epiphora with mucoid discharge; and 3=red conjunctiva, epiphora and feather matting, feather loss around periorbital ring, severe conjunctival oedema, and at least some chemosis or rhinitis. Both eyes were scored on days 0, 1, 3, 5, and 7.

M. gallisepticum *isolate*

The *M. gallisepticum* inoculum used in the experiment was the sixth *in vitro* broth (Frey's medium with 15% swine serum, modified from Kleven [1998]) passage from the original *M. gallisepticum* house finch isolate, ADRL 7994-1 (Ley et al. 1996). The viable count of this inoculum (ADRL 7994-1 6P) was 3.24 x 10^5 CFU/ml as determined by colony counts on agar from serial dilutions (Kollias et al. 2004). The *M. gallisepticum* utilized for assay development and sequencing was the seventh *in vitro* broth passage from *M. gallisepticum* house finch isolate, ADRL 7994-1.

DNA extraction and sample preparation

DNA extraction from conjunctival swab samples was carried out using a Qiagen DNeasy blood and tissue kit (Qiagen, Valencia, California, USA), following the manufacturer's recommended protocol for the purification of total DNA from animal tissues. The samples of experimentally infected house finches were diluted to a DNA concentration of 50 ng/5 µl for use in the qPCR. The DNA concentration was determined using a Bio-Rad SmartSpec™ 3000 (Bio-Rad, Hercules, California, USA) following the manufacturer's protocol. The samples of *M. gallisepticum*-negative birds were not further diluted because spectrophotometer readings of these samples were very low and accurate quantification of DNA in the samples was not feasible.

Preparation of DNA standards

A 303 base pair (bp) region of the *mgc2* gene was amplified from the genome of *M. gallisepticum* strain ADRL 7994.1 by PCR with previously published primer sequences (Garcia et al. 2005) using the following PCR parameters: 94°C for 3 min; followed by 35 cycles of 94°C

for 30 sec; 58°C for 30 sec, 72°C for 60 sec; followed by one cycle of 72°C for 10 min. A 1100 bp region of *rag-1* was amplified from house finch, American goldfinch (*Carduelis tristis*), house sparrow (*Passer domesticus*), and chicken (*Gallus gallus*) DNA with the R17 and R22 primers published by Groth and Barrowclough (1999). The PCR amplification parameters were: 5 min at 94°C; followed by 35 cycles of 94°C for 30 sec; 55°C for 40 sec, 72°C for 60 sec; followed by one cycle of 72°C for 10 min. A 119 bp fragment of the house sparrow 18S rRNA gene was amplified with conserved primer sequences (Lopez-Andreo et al. 2005) using the same PCR parameters as described for *rag-1*. The PCR products were cloned in the pCR®4-TOPO vector (Invitrogen, Carlsbad, California, USA) following the manufacturer's protocol. Random colonies were grown overnight in Luria-Bertani broth containing ampicillin, and plasmid DNA was extracted using a Qiagen MiniPrep kit (Qiagen) following the standard kit protocol. The insert was then sequenced (Automated 3730 DNA Analyzer; Applied Biosystems, Foster City, California, USA) and purified plasmid DNA from one clone containing the correct insert was used to construct the standard curves. For the *rag-1* assay standard, plasmid was harvested from a clone containing the house finch *rag-1* insert. The plasmid standard for the nitric oxide synthase 2 (*nos2*) assay was kindly provided by K.W. Jarosinski, Department of Microbiology and Immunology, Cornell University (Jarosinski et al. 2002). *M. gallisepticum* genomic DNA was extracted from the inoculum (ADRL 7994.1) utilized in this study to serve as an additional standard for the *mgc2* qPCR. Fresh 10-fold dilutions were made from standard stocks in DNase-free water.

Primer and probe design

A 303 bp sequence of the *mgc2* gene of isolate ADRL 7994-1 was aligned with *mgc2* sequences

of *M. gallisepticum* isolated from six house finches and one goldfinch from various locations and years. Primers and probes were designed with Primer Express (version 2.0; Applied Biosystems). Primers and probes for the real-time assays were synthesized by Integrated DNA Technologies (Coralville, Iowa, USA). Probes had 6-FAM on the 5′ end and BHQ-1 on the 3′ end—with the exception of the *nos2* probe, which had TET on the 5′ end and TAMRA on the 3′ end. Lasergene sequence analysis software (DNAstar, Madison, Wisconsin, USA) was utilized to create an alignment of *rag-1* sequences of two house finches, a goldfinch, a house sparrow and a chicken. Primers and probe were chosen to be in areas of 100% identity in the house finch and goldfinch to ensure primers anneal to areas conserved among house finches. Table 4.1 presents a list of the primer and probe sequences used in this study.

Real-time assays

The 20 µl reaction was performed in an ABI Prism 7500 (Applied Biosystems). The "fast" reaction utilized 10 µl TaqMan® Fast Universal PCR Master Mix (2x), No AmpErase® UNG (Applied Biosystems), 0.18 µl each of 100µM forward and reverse primers, 0.5 µl of 10 µM probe, 4.14 µl DNase-free water, and 5 µl sample. Samples were run in triplicate. Cycling parameters were 95°C for 20 sec and 40 cycles of 95°C for 3 sec and 60°C for 30 sec. The automatic threshold settings were used in analysis of samples. Samples were positive for *M. gallisepticum* if the cycle threshold (Ct) value was less than 40.

Nucleotide sequence accession numbers

Sequences can be accessed from GenBank (http://www.ncbi.nlm.nih.gov) with the following accession numbers: *M. gallisepticum* strain 7994-1 *mgc2* (EF462343), house sparrow 18S rRNA

gene (EF462342), and *rag-1* sequences from two house finches (EU165349, EU165350), a chicken (EU165351), a house sparrow (EU165352), and a goldfinch (EU165353). The *mgc2* sequences used in alignment of isolates from free-living finches included GenBank accession numbers AY556264, AY556259, AY556257, AY556245, AY556242, AY556240, and AY556233.

Statistical analysis

JMP (version 7.0; SAS Institute) was utilized for the least squares regression analysis presented later in Figure 2. We report coefficient estimates (β), *P* values, and R^2 values.

4.4 Results

Primers and probe

The ADRL 7994-1 partial *mgc2* sequence was found to be identical to that of the seven other house finch and goldfinch isolate sequences available in GenBank, and the real-time primers and probe (Table 4.1) were developed to amplify a 227 bp region within the 303 bp region. The approximate size was confirmed by running real-time products on an agarose gel (data not shown). A BLAST search with the *mgc2* real-time primers revealed significant alignments with a multitude of *M. gallisepticum* isolates, and no alignments with other *Mycoplasma* species. When the *mgc2* sequence of ADRL 7994-1 and several *M. gallisepticum* reference strains were compared, several nucleotide polymorphisms were present in the qPCR target region (data not shown).

Table 4.1: Real-time primer and probe sequences utilized in this study

Gene	Primer, probe	Sequence	Reference
MGC2	MGC2 forward	5'-GGTCCTAATCCCCAACAAAGAAT	Designed using
	MGC2 reverse	5'-CTTGGTTGGTTCATATTAGGCATTT	Primer Express
	MGC2 probe	5'-6-FAM-CCACAGGGCTTTGGTGGCCCA-BHQ-1	
18S	18S forward	5'-AGCCTGCGGCTTAATTTGAC	Lopez-Andreo *et*
rRNA	18S reverse	5'-CAACTAAGAACGGCCATGCA	*al*., 2005
	18S probe	5'-6-FAM-AGGATTGACAGATTGAG-BHQ-1	
NOS2	NOS2 forward	5'GAGTGGTTTAAGGAGTTGGATCTGA	Jarosinski *et al*.,
	NOS2 reverse	5'TTCCAGACCTCCCACCTCAA	2002
	NOS2 probe	5'TET–CTCTGCCTGCTGTTGCCAACATGC-TAMRA	
RAG-1	RAG-1 forward	5'TCAGGACTGCCACTCTCAATTG	Designed using
	RAG-1 reverse	5'CAAACCAAGGCTGCATCGTA	Primer Express
	RAG-1 probe	5'6-FAM-ACCCAGTAGACACAATTGCAAAGAGATTCCG-BHQ-1	

Standard curves

The *mgc2* standard was based on 10-fold serial dilutions of plasmid containing a 303 bp *mgc2* insert. The curve was created using 3.41×10^1 to 3.41×10^8 copy numbers. Serial 10-fold dilutions of the *M. gallisepticum* genomic DNA were used to create the curve using 1.00×10^0 to 1.00×10^6 copy numbers. The *rag-1* and 18S rRNA gene standards were based on 10-fold serial dilutions of plasmids containing the 1100 bp house finch *rag-1* and the 119 bp house sparrow 18S rRNA gene inserts, respectively. To generate the standard curves for *rag-1* and 18S rRNA, 3.76×10^1 to 3.76×10^7 and 1.40×10^1 to 1.40×10^7 copy numbers, respectively, were used. Standard curves were generated for each run. The following standard curve equations represent typical runs: *mgc2* plasmid, $y=-3.382x+39.98$, $R^2=0.998$; *M. gallisepticum* genomic, $y=-3.723x+39.885$, $R^2=0.996$; *rag-1*, $y=-3.504x+39.16$, $R^2=0.997$; and 18S rRNA gene, $y=-3.578x+46.741$, $R^2=0.987$.

Assay detection limit and reproducibility

The *mgc2* assay detection limit was determined using two standards, a plasmid standard and *M. gallisepticum* genomic DNA. The assay exhibited a detection limit of less than 14 copies per reaction using the plasmid standard and less than 10 copies per reaction using the genomic DNA standard. The inter-assay reproducibility of the *mgc2* plasmid standard and genomic DNA standard (Table 4.2) was determined using the mean Ct values from three independent runs, conducted on different days with new standard dilutions for each run. The intra-assay reproducibility of the *mgc2* standard curve (Table 4.2) is based on the mean Ct values from the standard curve run in triplicate. The linearity of the *mgc2* and *M. gallisepticum* genomic DNA standard curves is shown in Figure 4.1, with each curve representing the mean inter-assay Ct

values presented in Table 4.2. The data demonstrate that the *mgc2* assay is highly reproducible.

Table 4.2: Reproducibility and limit of detection of the standard curves used for *mgc2* assay

mgc2 plasmid standard						Genomic standard		
Inter-assay reproducibility			Intra-assay reproducibility			Inter-assay reproducibility		
Copies	Mean Ct	St. dev	Copies	Mean Ct	St. dev	Copies	Mean Ct	St. dev
$1.40x10^{8}$	12.52	0.452	$1.40x10^{8}$	12.92	0.03	$1.00x10^{6}$	18.39	1.025
$1.40x10^{7}$	15.70	0.352	$1.40x10^{7}$	15.99	0.05	$1.00x10^{5}$	21.87	1.049
$1.40x10^{6}$	19.18	0.354	$1.40x10^{6}$	19.59	0.05	$1.00x10^{4}$	25.58	1.097
$1.40x10^{5}$	22.85	0.344	$1.40x10^{5}$	23.01	0.15	$1.00x10^{3}$	29.18	1.143
$1.40x10^{4}$	26.36	0.290	$1.40x10^{4}$	26.5	0.16	$1.00x10^{2}$	33.05	1.393
$1.40x10^{3}$	29.72	0.431	$1.40x10^{3}$	30.04	0.21	$1.00x10^{1}$	36.61	0.795
$1.40x10^{2}$	32.86	0.313	$1.40x10^{2}$	33.12	0.2	$1.00x10^{0}$	40.00	-
$1.40x10^{1}$	34.91	0.653	$1.40x10^{1}$	35.5	1.17			
$1.40x10^{0}$	40.00	-						

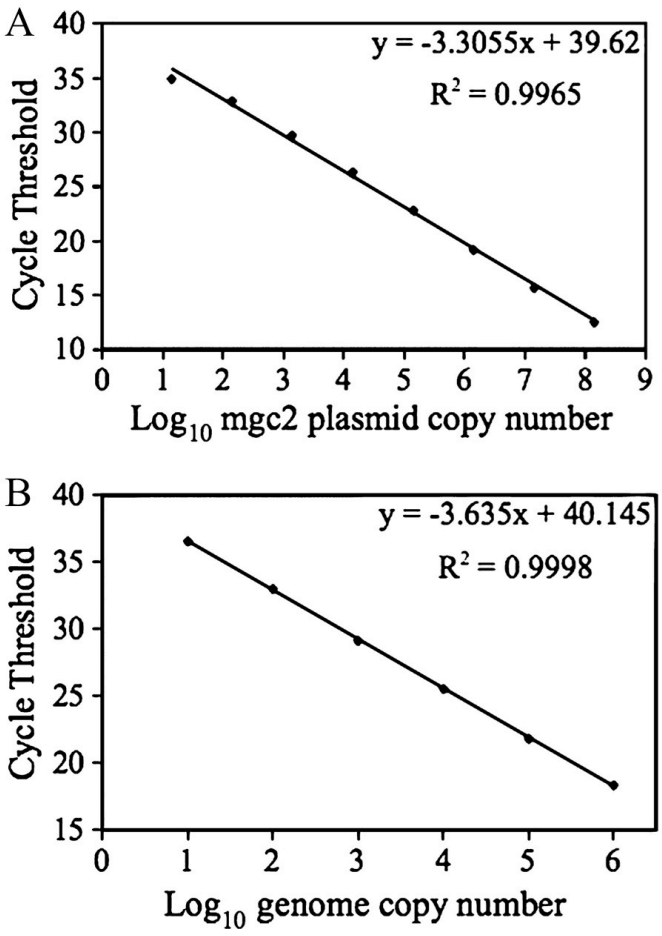

Figure 4.1. *Mgc2*-quantitative PCR: linearity of standard curve. A: Standard curve based on *mgc2* plasmid. B: Standard curve based on *M. gallisepticum* genomic DNA.

Control samples

Sensitive tests for the presence of *M. gallisepticum*-specific antibodies in passerine birds are lacking, and therefore it is not possible to obtain absolute confirmation that free-living passerine birds are truly negative for *M. gallisepticum*. Thus, we first confirmed that the *mgc2* assay tested negative with avian DNA by using *M. gallisepticum*-negative chicken swab samples. The 10 chickens and 10 house finches, all free of *M. gallisepticum* infection based on the absence of antibodies, were also negative using *mgc2* real-time PCR with Ct values >40. *Nos2* was used as a host DNA control for the chicken swab samples to ensure DNA was efficiently extracted. The *Nos2* copy number ranged from 363 to 40 000 copies/5 µl, with a mean copy number of 6509 and a standard deviation of 9177 copies. *Rag-1* was used as the house finch DNA control. The *Rag-1* copy number ranged from 13 to 1520 copies/5 µl, with a mean copy number of 255 and a standard deviation of 470 copies.

Quantification of M. gallisepticum *load in conjunctival samples of five infected house finches*

After experimental infection with *M. gallisepticum*, Bird 5 showed clinical signs by day 3, and Birds 2, 3, and 4 showed signs by day 7. Bird 1 did not develop clinical signs. Figure 4.2A illustrates the mean $\log_{10}$ of total *M. gallisepticum* eluted from the swab as a function of time p.i. in birds that developed clinical signs (Birds 2, 3, 4, 5). Total *M. gallisepticum* eluted from the swab increases with time p.i. ($\beta=0.985$, $P<0.0001$, $R^2=0.80$). In Figure 4.2B, the mean $\log_{10}$ of *M. gallisepticum* copy number per house finch cell is plotted as a function of time p.i. in birds that developed clinical signs. *M. gallisepticum*/host cell increases with time p.i. ($\beta=0.450$, $P<0.0001$, $R^2=0.84$). For each bird, the mean value of the right and left eyes was used to construct the plots in Figure 4.2.

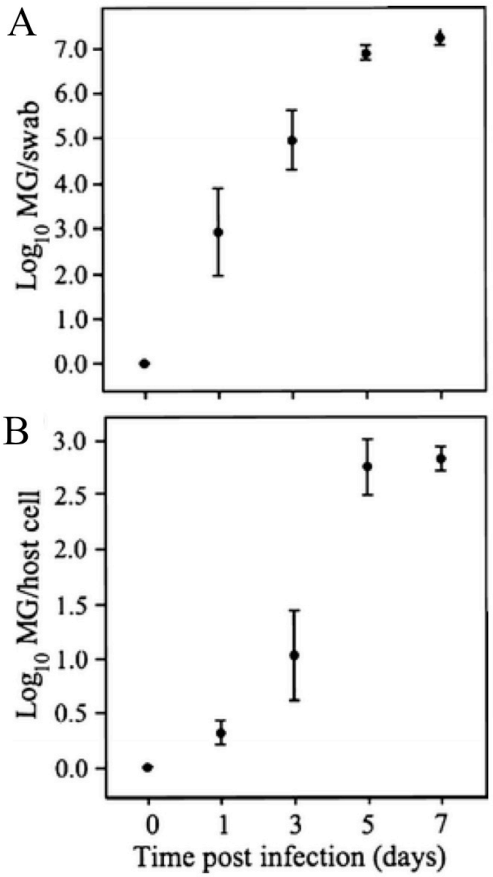

Figure 4.2. *M. gallisepticum* conjunctival load post-infection, measured by quantitative PCR. A: Mean *M. gallisepticum* (*MG*) load present in total swab samples of the conjunctiva of four inoculated house finches that developed clinical conjunctivitis, as a function of time p.i., with standard error bars. B: Mean *M. gallisepticum* (*MG*) load shown as the $\log_{10}$ value of *mgc2* copy number per host cell, with standard error bars.

A summary of maximal *M. gallisepticum* load and eye score at day 7 post-infection is also presented in Table 4.3, showing data without log transformation. Figure 4.3 shows individual data for each bird, with the *M. gallisepticum* load and eye score plotted over the 7 days p.i. Bird 5 exhibited the earliest eye score at 3 days p.i. and also had the highest *M. gallisepticum* load. Although there is variation among individuals, these data show that the right and left eyes of individual birds experience similar *M. gallisepticum* load. Figure 4.3 also illustrates that while birds had similar *M. gallisepticum* loads throughout the 7-day period (e.g. Birds 2 and 5), the clinical signs developed at different time points. There are three data points excluded from the analysis. Samples from Bird 1 (day 3, right eye) and Bird 4 (day 7, right eye) could not be analysed because of insufficient amount of DNA in the sample. It was also suspected that the sample of Bird 1 at day 0 (left eye) was contaminated during the DNA extraction process, and this data point was not included in the graph.

Table 4.3. Summary of maximal mycoplasmal load and eye score at day 7 post infection. MG = *M. gallisepticum*

Bird	Day 7 eye score	Maximum MG load (MG/host cell)	Day of maximum MG load
1	0	16	7
2	1	2079	5
3	1	400	7
4	1	1328	7
5	2	3359	5

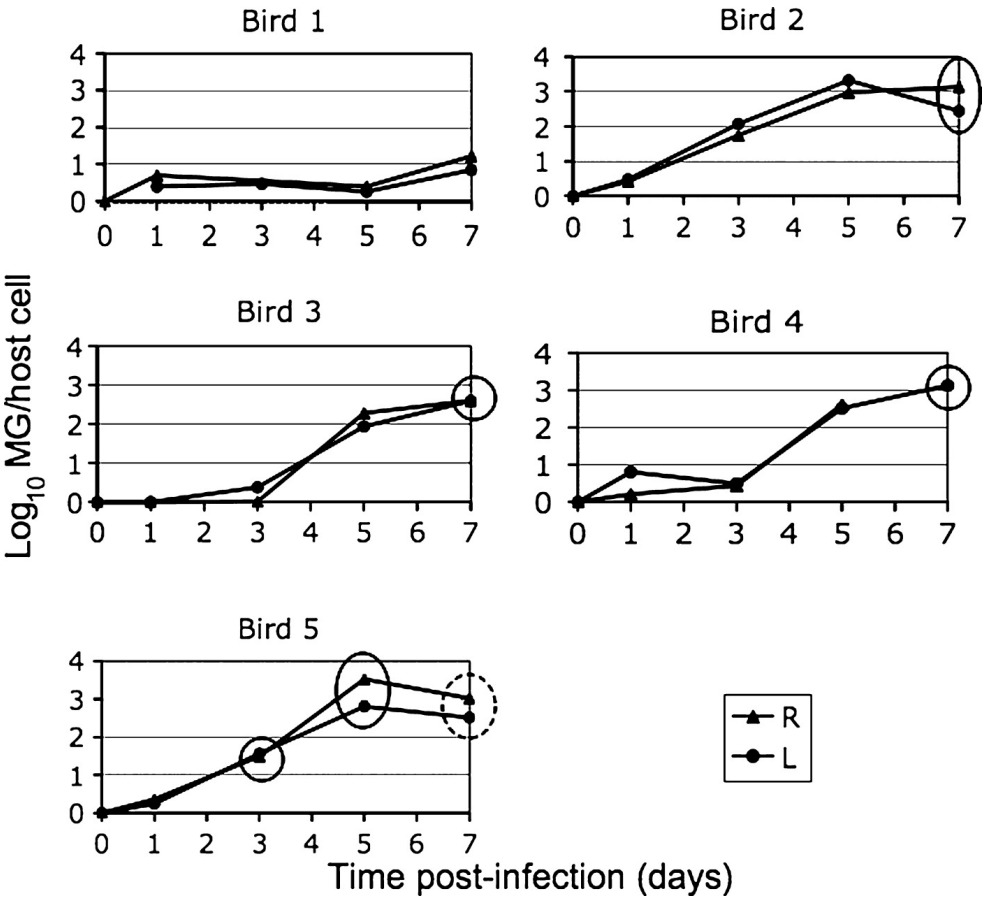

Figure 4.3. Data from individual birds showing *M. gallisepticum* (*MG*) load over time and occurrence of conjunctivitis. Solid circles, an eye score of 1 on the day of conjunctival sampling; dashed circle, a more severe eye score of 2. R, right eye; L, left eye. Eye score code: 0=no visible inflammation; 1=pink conjunctival discolouration and slight periorbital oedema, 2=pink conjunctival discolouration, slight to moderate periorbital oedema, and epiphora with mucoid discharge.

Host control genes

A BLAST search of the house sparrow 18S rRNA gene sequence confirmed that it had 100% sequence identity to many other avian species, including chicken. Thus, it was an appropriate standard in the house finch 18S rRNA gene qPCR assay. Among the house finches infected with *M. gallisepticum*, the average 18S rRNA gene copy number ranged from 1.57×10^2 to 1.40×10^8 (mean=$2.40 \times 10^6 \pm 1.82 \times 10^7$) in 50 ng DNA. House finch *rag-1* copy numbers were used to standardize the *M. gallisepticum* conjunctival load. Since *rag-1* is present in two copies per diploid cell, the number of host cells in each sample was calculated by dividing the *rag-1* copy number by two. In samples from experimentally infected birds (50 ng DNA/assay), the house finch cell number ranged from 7 to 2893 (mean=212±487).

4.5 Discussion

We have developed a TaqMan®-based assay to detect *M. gallisepticum* in conjunctival swab samples of experimentally infected house finches. The standard curve was reproducible both between runs and within runs. The detection limit of the *mgc2* assay was examined using two standards: *M. gallisepticum* genomic DNA extracted from culture, and a plasmid-based standard consisting of a partial *mgc2* gene inserted into a bacterial vector. The test has a detection limit of less than 14 copies per reaction when tested with the plasmid standard and less than 10 copies per reaction when tested with the genomic DNA standard. All *M. gallisepticum*-negative birds (10 chickens and 10 house finches) resulted in a negative *mgc2* assay result.

Our test also includes an internal control gene. The occurrence of conjunctivitis in infected finches makes the use of a host gene even more imperative, as host cells are being actively recruited to site of infection and may influence absolute quantity of the sample obtained.

The evidence that *M. gallisepticum* adheres to host cells and has the ability to invade cells further underscores the importance measuring the number of host cells collected in the conjunctival swab samples. Because there are potentially hundreds of 18S rRNA gene copies in the house finch genome, we decided to explore single-copy gene alternatives and found *rag-1* to be an acceptable host control gene that allows for quantification of host cells in the conjunctival samples. Our *rag-1* results demonstrate that host cell quantity varies quite substantially between conjunctival samples. The use of *rag-1* ensures that variation in samples due to swabbing method, infection status (e.g. birds with and without conjunctivitis), and any differences in DNA extraction methods are taken into account.

After experimental inoculation, *M. gallisepticum* levels in the house finch conjunctiva increase exponentially over the 7-day period p.i. The bird with the most severe conjunctivitis experienced the highest level of *M. gallisepticum* and the bird that did not develop conjunctivitis had a very low level of *M. gallisepticum*. Thus, it appears that development of conjunctivitis may correlate with *M. gallisepticum* load.

In summary, our real-time assay can provide sensitive detection of *M. gallisepticum* in conjunctival samples of house finches. The assay allows for the quantification of *M. gallisepticum* genome copy number while controlling for intrinsic differences that exist as a result of variations in sampling method and host inflammatory response. We hope this assay will serve as a valuable tool for future studies of *M. gallisepticum* pathogenesis and transmission in house finches.

Acknowledgements

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CHAPTER 5

Production of house finch (*Carpodacus mexicanus*) IgA specific anti-sera and its application in immunohistochemistry and in ELISA for detection of *Mycoplasma gallisepticum*-specific IgA*

* Previously published: Grodio, J.L., Buckles, E.L., and K.A. Schat. 2009. Production of house finch (*Carpodacus mexicanus*) IgA specific anti-sera and its application in immunohistochemistry and in ELISA for detection of *Mycoplasma gallisepticum*-specific IgA. *Veterinary Immunology and Immunopathology*. 132: 288-294. Reproduced with permission (license # 2987150532346).

5.1 Abstract

The IgA antibody response plays a vital role in mucosal immunity because it functions to neutralize pathogens at the mucosal surface and thus impedes attachment to underlying tissues. Although the importance of IgA in the mucosal immunity of galliform birds has been established, studies examining IgA-based immunity in passerine birds are lacking, perhaps due in part to the absence of reagents that can detect passerine IgA. A 469 base pair region of the house finch (*Carpodacus mexicanus*) IgA heavy chain was PCR-amplified from spleen cDNA and sequenced. The predicted amino acid sequence was found to share 55% and 46% identity with the IgA heavy chain of mallard (*Anas platyrhynchos*) and chicken (*Gallus gallus*), respectively. The heavy chain fragment was produced using a bacterial expression system and purified. Rabbit anti-sera were generated against the recombinant protein. The anti-sera reacted with a single house finch serum protein (~50–55 kDa) in Western blot. The anti-sera were used to identify plasma cells in the Harderian gland and conjunctiva of house finches with conjunctivitis associated with *Mycoplasma gallisepticum* infection. The anti-sera were also utilized in an ELISA to detect *M. gallisepticum*-specific IgA antibodies in lachrymal samples of infected finches.

5.2 Introduction

Secretory IgA (sIgA) plays a vital role in mucosal immunity by neutralizing pathogens at mucosal surfaces. While IgA antibody response of domestic avian species, particularly the chicken, has been well studied (Barbour et al. 1988, Bencina et al. 1991, Suresh et al. 1994, Toro et al. 1994), comparatively little is known about sIgA antibody responses of passerine birds. The development of class-specific monoclonal antibodies to chicken IgA has facilitated past research. Many of these monoclonal antibodies developed against chicken immunoglobulins, however, show poor cross-reactivity with those of other avian species, especially to those distantly related to the chicken (Jeurissen and Janse 1998, for review see Schultz and Magor 2008).

Few reagents have been developed to detect immunoglobulins of passerine birds. Commercial polyclonal antibodies are available for the detection of bird IgM (Immunology Consultants Laboratory, Newberg, OR) and IgY (Bethyl Laboratories, Montgomery, TX). These polyclonal antibodies were generated by immunizing rabbits and goats with immunoglobulin from at least one species of passerine bird (e.g., robin and crow IgM and sparrow IgY). To date, no reagents have been developed against IgA from a passerine bird.

This chapter describes the production and characterization of a polyclonal reagent specific for the house finch (*Carpodacus mexicanus*) immunoglobulin alpha heavy chain. To validate the anti-sera, I utilized serum, lachrymal samples, and tissues samples of house finches infected with the pathogenic bacterium *Mycoplasma gallisepticum*, which causes conjunctivitis in free-living finches (Ley et al. 1996, Kollias et al. 2004).

5.3 Materials and methods

RNA isolation and cDNA synthesis

House finch spleens were provided by K. Dhondt (College of Veterinary Medicine, Cornell University) and immediately placed in 500 µL PBS after collection. Total cellular RNA was isolated using RNA STAT 60 (Tel-test Inc., Friendswood, TX) following the manufacturer's instructions. RNA was re-suspended in 50 µL RNase-free water and DNase-treated with Turbo DNA-free system (Ambion Inc., Austin, TX) following manufacturer's instructions. RNA samples were stored at −80°C.

The reverse transcriptase reaction was carried out using GeneAmp RNA PCR Core Kit (Applied Biosystems, Foster City, CA) following manufacturer's instructions. The 20 µL reaction contained 0.5 µg total cellular RNA, 5 mM $MgCl_2$, 1× PCR buffer II, 1 U/µL RNase inhibitor, 2.5 U/µL MuLV reverse transcriptase, 2.5 µM random hexamers, and 1 mM each of dGTP, dATP, dTTP, and dCTP. The reaction was incubated at 25°C for 10 min, 42°C for 20 min, and 99°C for 5 min with the GeneAmp PCR system 9700 (Applied Biosystems).

PCR and cloning of partial house finch IgA (alpha) heavy chain cDNA sequence

Mallard duck (*Anas platyrhynchos*) and chicken (*Gallus gallus*) IgA heavy chain cDNA sequences available on Genbank (accession numbers U27222 and S40610, respectively) were aligned using BLASTN (NCBI) and primers were designed in regions of similarity (forward: 5’ TCTGCGCCGAGGAGTGGAAC 3’ and reverse: 5’AGGATCACCGTGACGTTGACGTTG 3’)

The house finch alpha heavy chain was amplified from cDNA by PCR in a reaction that contained: 2 µL of the RT reaction, 4.8 µL 10× Gold buffer, 3.6 µL of 25 mM $MgCl_2$, 0.8 µL of

10 mM dNTP, 0.5 μL each of forward and reverse primers and AmpliTaq Gold polymerase (Applied Biosystems). The reaction was incubated at 94°C for 5 min; 35 cycles of: 94°C for 30 s, 50°C for 40 s, and 72°C for 60 s; and 72°C for 10 min. The resulting PCR product was cloned in the pCR®4 TOPO vector (Invitrogen, Carlsbad, CA) following the manufacturer's protocol with TOP10 cells. Random colonies were grown overnight in LB broth containing ampicillin, and plasmid DNA was extracted using a Qiagen MiniPrep kit (Qiagen, Valencia, CA) following the manufacturer's protocol. The insert was sequenced (Automated 3730 DNA Analyzer, Applied Biosystems) and Lasergene sequence analysis software (DNAstar, Madison, WI) was utilized to determine the reading frame and predicted amino acid sequence. The sequence was submitted to Genbank (EU736222).

Construction of house finch IgA expression plasmid

A blunt-ended sequence was generated using cloned Pfu DNA polymerase in a reaction recommended by the manufacturer (Stratagene, La Jolla, CA) using primers designed to add the 5' sequence required for insertion into the vector, an ATG start codon, and a stop codon (forward: 5' CACCTGCGCCGAGGAGTGGAAC 3', reverse: 5' CTAGAGGATCACCGTGACGTTGACGTTG 3'). The reaction was incubated at 95°C for 45 s; 30 cycles of: 95°C for 45 s, 57°C for 45 s, 72°C for 60 s; and 72°C for 10 min. The resulting PCR product was gel-purified using a QIAquick gel extraction kit (Qiagen) and cloned into the pET100 vector (Invitrogen) using manufacturer's protocol. TOP10 *E. coli* were transformed following the manufacturer's instructions and plasmid DNA was isolated using a Qiagen MiniPrep kit (Qiagen).

Expression and purification of partial house finch IgA alpha heavy chain

Two vials of BL21 Star (DE3) *E. coli* (Invitrogen) were transformed with 10 ng of plasmid DNA, incubated on ice for 30 min, heat-shocked at 42°C for 30 s, and tubes immediately transferred to ice. 250 μL of SOC media was added to each tube and incubated at 37°C for 30 min and the mixture was added to 10 mL of LB broth with ampicillin and 1% glucose and grown overnight at 37°C. 250 mL of prewarmed LB/ampicillin/glucose media was inoculated with 10 mL of the overnight culture and grown at 37°C until an OD600 of 0.6 was reached. 1 mM isopropylthio-β-galactoside (Invitrogen) was added to induce expression of the construct and the culture was incubated for an additional 1.5 h at 37°C. The culture was pelleted at 3750 × g for 25 min. Protein was harvested under denaturing conditions as per manufacturer's instructions using the Ni-NTA Fast Start kit (Qiagen).

The purified product was analyzed by a total protein blot using Ponceau S stain (0.1% Ponceau S in 5% acetic acid). Protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, IL) following the manufacturer's "test tube procedure" protocol.

Production of anti-sera

Two New Zealand White rabbits were immunized with the recombinant protein. The immunization protocol was carried out by Cocalico Biologicals (Reamstown, PA). Both pre-immunization sera and anti-sera were collected.

Western blotting

House finch serum and recombinant IgA were prepared under denaturing conditions for SDS-PAGE. Recombinant protein (0.70 mg/mL) was diluted 1:300 in PBS. House finch serum was

diluted 1:100 in PBS. Samples were diluted 1:1 with sample buffer (4% SDS, 20% glycerol, 50 mM Tris, pH 6.8, 0.1% bromophenol blue, 10% 2-mercaptoethanol) and separated on a polyacrylamide gel containing a stacking gel of 5% and a separating gel of 10%. SeeBlue® Plus2 pre-stained standard (Invitrogen) was run concurrently with the samples. Proteins were transferred to 0.45 μM nitrocellulose membranes (Bio-Rad, Hercules, CA) using transfer buffer (20 mM Tris, 150 mM glycine, and 20% methanol) at 200 V and 4°C. After transfer, the membrane was stained with Ponceau S (0.1% Ponceau S in 5% acetic acid) to visualize transfer. Membranes were de-stained by rinsing in distilled water and incubated in blocking buffer (Tris-buffered saline with 1% Tween 20 [TBS-T] plus 5% non-fat dry milk [Nestlé, Vevey, Switzerland]) for 2 h at room temperature. After washing in TBS-T, the blots were incubated with either the pre- or post-immunization sera. Sera from rabbit 1 were diluted 1:2000, while sera from rabbit 2 were diluted 1:4000 in TBS-T with 5% non-fat dry milk and incubated overnight at 4°C on a rocker. After washing in TBS-T, the blots were incubated with HRP-conjugated goat anti-rabbit IgG (Bethyl Laboratories, Montgomery, TX) diluted 1:10,000 in TBS-T with 5% non-fat dry milk for 1 h at room temperature. Blots were washed with TBS-T and ECL Western blotting substrate (Pierce) was applied as recommended by the manufacturer, incubated for 5 min, covered with plastic wrap and exposed to film (Amersham Hyperfilm ECL, GE Healthcare, Piscataway, NJ) in a dark room.

Tissue sources

Harderian gland and conjunctival tissues were collected from house finches, house sparrows (*Passer domesticus*) and American goldfinches (*Carduelis tristis*) infected with *M. gallisepticum* 6 weeks post-infection. One-year-hatchling birds were trapped in Tompkins County, NY (42°

51' N, 76° 34' W) during October and November 2006 using mist nets under permits (LCP 99-039) from New York State Department of Environmental Conservation (Albany, NY) and a federal collecting permit (PRT 802829). As part of a larger study (K. Dhondt et al. unpublished) the birds were infected with *M. gallisepticum* by applying 50 μL of inoculum (3.24×10^5 colony forming units/mL) to each eye.

Preparation of samples for immunohistochemistry

After overnight fixation of whole birds in 10% neutral buffered formalin, the Harderian glands were dissected from the eye orbit, along with surrounding ocular tissues. The fixed tissues were embedded in paraffin, serially sectioned at 5.0 μM, mounted on slides, and used for hematoxylin-eosin (H&E), immunoperoxidase, or double immunofluorescence staining.

Immunoperoxidase staining

Sections were de-waxed in xylene, rehydrated through a series of solutions of decreasing ethanol concentrations (100%, 95%, 70%), and placed in water. To block for endogenous peroxidase, the slides were immersed in 0.05% hydrogen peroxide in methanol for 10 min, washed in 70% ethanol and placed in PBS. The anti-sera were initially tested without antigen retrieval, however, at a later date an antigen retrieval step was added by subjecting the sections to microwaving at high power (800 W) for 20 min in 0.01 M sodium citrate buffer, pH 6.0. After washing in PBS, the sections were incubated in 10% normal goat serum and 10% non-fat dry milk for 20 min at room temperature in a humid chamber. The blocking solution was blotted off and rabbit anti-house finch IgA serum was diluted 1:120 in PBS with casein (Vector Laboratories, Burlingame, CA) and incubated in a humid chamber for 2 h at 37°C. After washing in PBS, the sections were

incubated with a biotinylated goat anti-rabbit IgG (Vector Laboratories) diluted 1:200 in PBS for 20 min at room temperature in a humid chamber. After washing in PBS, the sections were incubated with HRP-streptavidin (Zymed-Invitrogen, Carlsbad, CA) diluted 1:200 in PBS for 20 min at room temperature. After washing in PBS, AEC chromogen/substrate solution (Zymed-Invitrogen) was applied and after appropriate color developed the reaction was stopped with distilled water. The sections were counterstained using Gill's #2 hematoxylin for 30 s, rinsed with water, and mounted.

Double immunofluorescence

The sections were blocked with 10% donkey serum and 10% non-fat dry milk in PBS-0.05% Tween and incubated with goat anti-bird IgG (Bethyl Laboratories) diluted 1:300 and anti-house finch IgA diluted 1:20. Biotin-labeled donkey anti-goat IgG (Jackson ImmunoResearch, West Grove, PA) diluted 1:200 and FITC labeled donkey anti-rabbit IgG (Jackson ImmunoResearch) diluted 1:80 were applied, followed by incubation with streptavidin Texas Red (Vector Laboratories) diluted at 1:200. The sections were mounted with Vectashield mounting medium with DAPI (Vector Laboratories).

Experimental infection and collection of lachrymal fluid

One-year-hatchling house finches were captured with appropriate permits (Montgomery Co., VA: VDGIF [031626], Tompkins Co., NY: USGS Bird Banding Lab [23513], CA: [SCL\P 802041-01]). These birds were inoculated with *M. gallisepticum* as part of a larger study (Hawley et al. 2010), and lachrymal samples were collected pre-inoculation and weekly post-infection as described below. We used a subset of 119 lachrymal samples (sample sizes: week

0 = 37; week 1 = 26; week 2 = 16; week 3 = 24; week 4 = 16) to test whether the anti-sera could be used in ELISA to detect *M. gallisepticum*-specific IgA in lachrymal fluid.

Schirmer tear test strips (Schering-Plough Animal Health, Union, NJ) were modified to fit into the conjunctiva of a house finch. Two cuts were made aseptically to form a rectangle of 3 mm × 30 mm. When applied to the 3 mm end of the strip, 1 μL saline absorbs onto a 3 mm × 4 mm area on the strip. This makes it possible to quantify the amount of lachrymal fluid collected from each bird.

For each individual sample, the 3 mm end of the strip was held in the conjunctiva for 15 s. The fluid boundary mark was recorded and the strip was immediately placed into 250 μL of PBS/0.05% Tween 20. The tube was vortexed vigorously, the tear strip was discarded and the sample was stored at −28°C.

ELISA

Idexx FlockChek MG antibody ELISA kit (Idexx, Westbrook, ME) was used to measure *M. gallisepticum*-specific IgA in house finch lachrymal samples by modifying the manufacturer's protocol. First, 300 μL of 1% BSA (Pierce 10× BSA, Thermo Fisher Scientific, Rockford, IL) in PBS-0.05% Tween 20 was added to each well and plates were incubated at room temperature for 40 min. After washing three times with 350 μL of PBS-T (ELx50 Auto Strip Washer, Bio-Tek, Winooski, VT), lachrymal samples (0.3 μL lachrymal fluid in 100 μL PBS-T) were added to the wells and plates were incubated for 1 h at room temperature. After washing with PBS-T, rabbit anti-house finch IgA serum (diluted 1:200 in PBS-T) was added to each well and the plates were incubated for 1 h at room temperature. After washing, goat anti-rabbit IgG (Bethyl Laboratories) diluted 1:60,000 in PBS-T was added and the plates were incubated for 40 min at room

temperature. After washing, 100 μL of TMB (included in Idexx kit) was applied to the wells and plates were incubated for 15 min followed by the addition of 100 μL of stop solution. The absorbance was read at 630 nm (ELx800 Universal Microplate Reader, Bio-Tek). Samples were run in duplicate and the average was calculated for further analysis. The authors were blinded to the infection status of the samples.

Statistical analysis

A one-way ANOVA was performed on ELISA data using JMP statistical analysis software (version 7.0, SAS Institute).

5.3 Results and discussion

House finch IgA heavy chain sequence

A 469 base pair region of house finch alpha heavy chain was amplified from spleen cDNA. Based on the predicted sequence of the chicken IgA constant region (Mansikka 1992), the forward primer targets a sequence within constant region 3 and the reverse primer targets sequences within constant region 4 of the chicken IgA heavy chain. The predicted amino acid sequence of the cloned house finch IgA constant region shares 46% and 55% amino acid sequence identity with chicken and duck, respectively. Figure 5.1 shows the alignment of house finch, chicken, and mallard duck amino acid sequences. The four cysteine residues present in the duck and chicken sequences were also conserved in the house finch sequence.

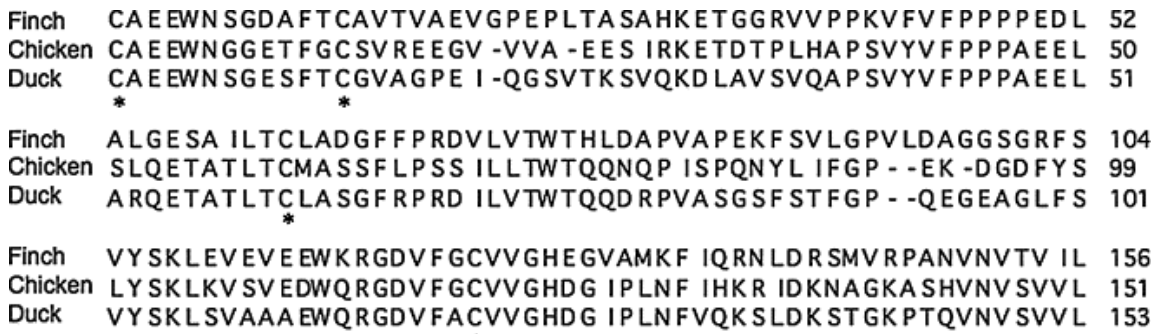

Figure 5.1. Alignment of the partial IgA heavy chain sequence of the house finch, chicken, and mallard duck. Conserved cysteine residues are marked with an asterisk.

Specificity of the anti-sera

The house finch IgA cDNA sequence was inserted into an expression vector, expressed in *E. coli*, and a 21 kDa recombinant house finch IgA protein was produced and purified using a 6-histidine tag added to the recombinant protein (Figure 5.2A). This recombinant protein was used for anti-sera production. The anti-sera reacted with a single house finch serum protein in Western blot (Figure 5.2B). Used at the same dilution of the anti-sera, the pre-immune sera showed no background staining. The protein band was ~50–55 kDa, approximately equal to the size of the alpha heavy chain of the duck (Oliveira et al. 2006). The anti-sera did not react with the light chain, which has an expected size of ~22 kDa (Oliveira et al. 2006, Davison et al. 2008).

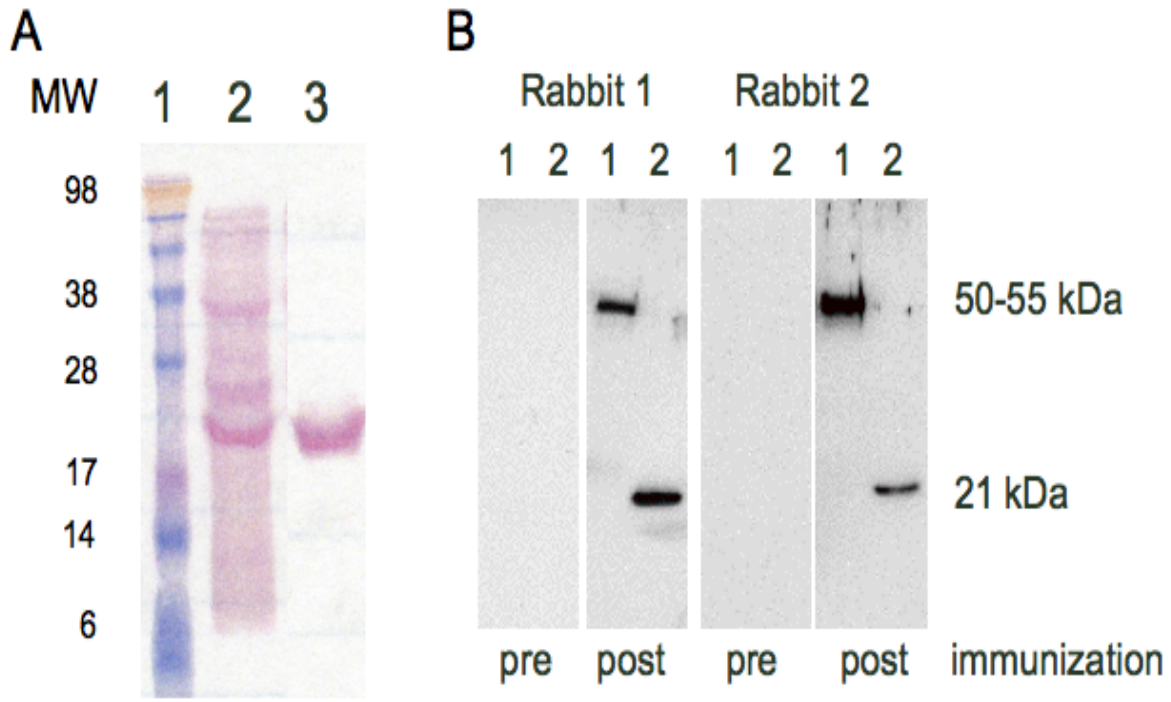

Figure 5.2. (A) Total protein blot showing purification of recombinant IgA. After expression of house finch IgA, *E. coli* cells were lysed. Recombinant IgA (~21 kDa) was purified from other proteins in the supernatant by use of a chromatography column that binds specifically to the 6-histidine tag present on the recombinant IgA. Lane 1: SeeBlue® Plus2 Pre-stained standard ladder, labeled with approximate molecular weight (MW) in kDa, lane 2: *E. coli* supernatant, lane 3: purified recombinant IgA. (B) Western blot using anti-house finch IgA sera. House finch sera were run in lane 1 and recombinant IgA protein was run in lane 2 under denaturing conditions. The blots were then incubated with either pre- or post-immunization rabbit serum. After immunization, both rabbits produced antibodies that reacted to the house finch IgA heavy chain (50‑55 kDa) and the recombinant protein (21 kDa).

The Harderian gland was chosen for immunohistochemical analysis because in birds it contains a large population of IgA-secretion plasma cells (Olah et al. 1992, Ohshima and Hiramatsu 2002, Oliveira et al. 2006, Khan et al. 2007). When viewed with H&E stain, the house finch Harderian glands contained many plasma cells with eccentrically located nuclei, abundant cytoplasm, and prominent Golgi (Figure 5.3). Using immunoperoxidase staining, the anti-sera labeled plasma cells in the house finch Harderian gland (Figure 5.4) and in the conjunctiva of house finches infected with *M. gallisepticum* (Figure 5.5). When pre-immunization sera were used in place of the anti-sera, no staining was present (Figure 5.4A and C). Many IgA+ cells were located in the lamina propria of the Harderian gland (Figure 5.4B). In some areas, the luminal linings of the ducts were IgA+ and occasionally, an IgA+ cell was found within the duct epithelium (Figure 5.4D). Oliveira et al. (2006) also documented intraepithelial plasma cells in the Harderian gland of the duck. Without antigen retrieval, IgA+ cells often stained with a single red spot inside the cytoplasm (Figure 5.4D), whereas with antigen retrieval by microwave treatment the cytoplasm stained uniformly IgA+. Oliveira et al. (2006) used a similar antigen retrieval step to visualize plasma cells in the Harderian gland of the duck. The anti-sera also stained cells in the Harderian gland of the house sparrow and American goldfinch (Figure 5.6). Thus, it is expected that this reagent will be appropriate for use in other related species.

There are also IgY+ plasma cells in the lamina propria of the avian Harderian gland (Ohshima and Hiramatsu 2002, Oliveira et al. 2006, Khan et al. 2007). To ensure that the anti-sera were staining IgA+ plasma cells specifically, double immunofluorescence was carried out with goat anti-bird IgY affinity-purified polyclonal reagent and the house finch IgA anti-sera. The double immunofluorescence of plasma cells in the Harderian gland (Figure 5.4E) and conjunctival tissue (Figure 5.4F) showed labeling of IgY+ cells (red) and IgA+ cells (green) with

little co-labeling, thus confirming the specificity of the anti-house finch IgA sera.

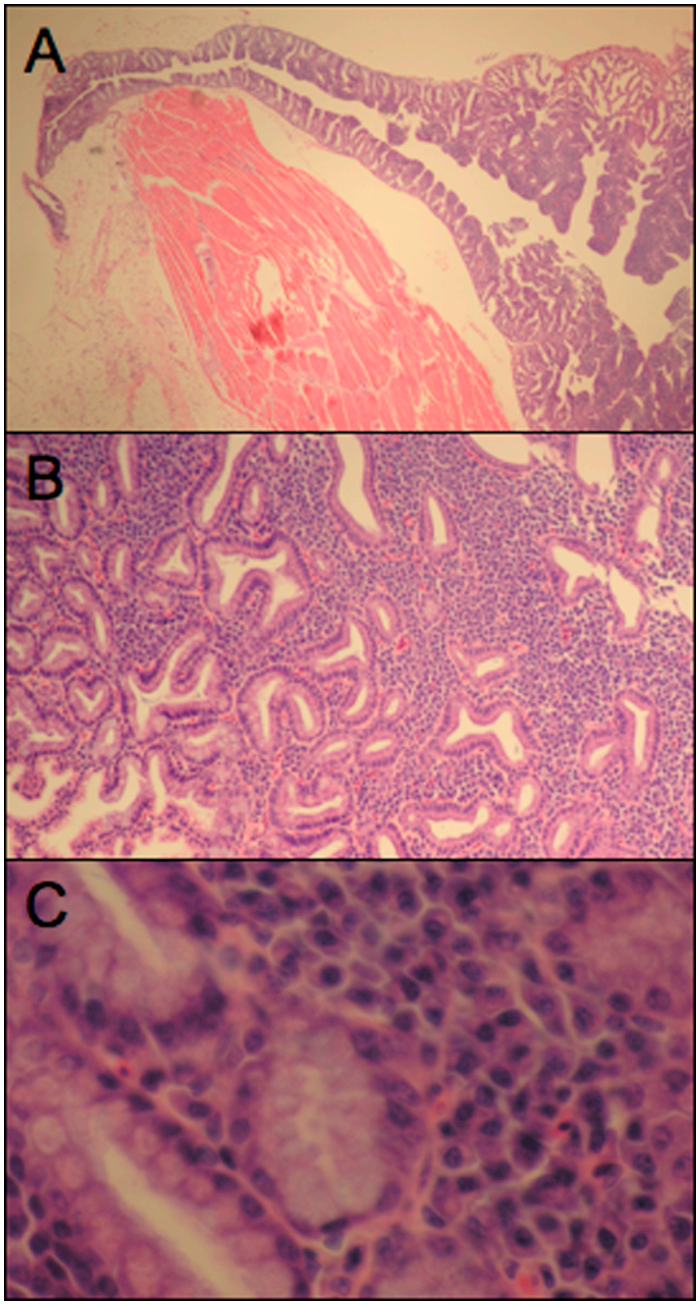

Figure 5.3. Histology of house finch Harderian gland. A. Partial view (40×) of the house finch Harderian gland and adjacent ocular muscle, showing duct of Harderian gland. B. Harderian gland (100×) showing acini and a highly cellular lamina propria. C. Harderian gland (600×). Beneath the glandular epithelium lie lymphocytes, many with cellular characteristics common to plasma cells (prominent Golgi, eccentric nucleus, abundant cytoplasm).

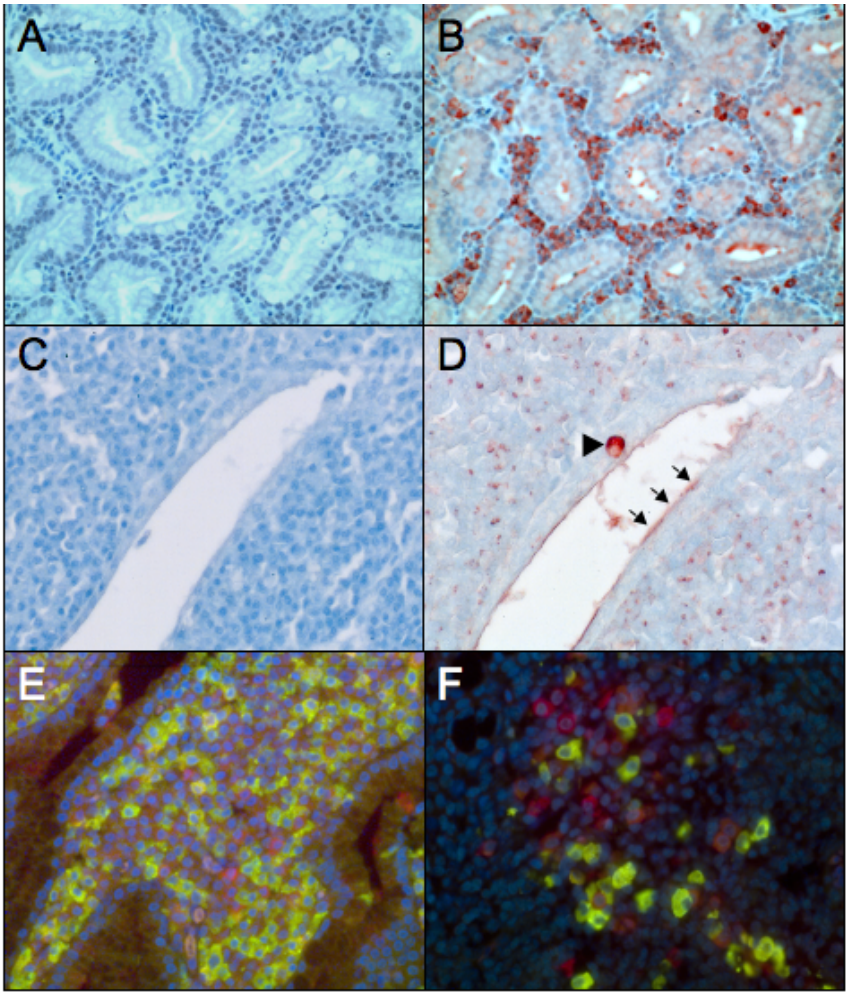

Figure 5.4. Immunoperoxidase staining of IgA+ plasma cells in the house finch Harderian gland using the pre-immunization sera (A and C) or rabbit anti-house finch IgA sera (B and D). In (D) arrowhead points to intraepithelial IgA+ plasma cell, arrows point to IgA+ luminal lining. Antigen retrieval was used for (A and B) (resulting in complete cytoplasmic staining), but not in (C and D) (resulting in IgA+ spots in cytoplasm). Immunofluorescence staining of IgA+ (green) and IgY+ (red) plasma cells in the Harderian gland (E) and conjunctiva of a finch with *M. gallisepticum*-associated conjunctivitis (F). Nuclei are stained blue. Magnification (A–F) 400×.

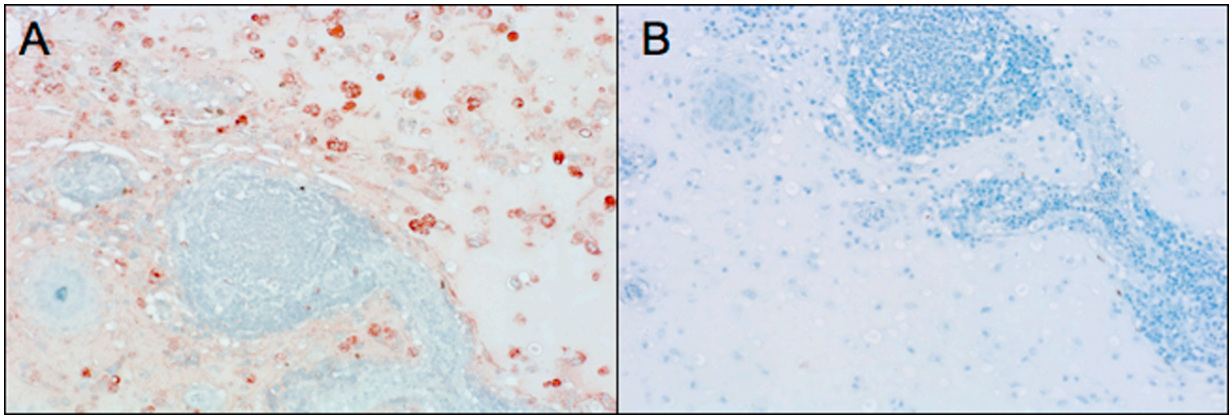

Figure 5.5. Immunoperoxidase staining of IgA+ cells in the conjunctiva of a house finch infected with *Mycoplasma gallisepticum* (200×). Stained with anti-house finch IgA sera (A) or pre-immunization control sera (B), followed by detection using biotin-labeled goat anti-rabbit IgG.

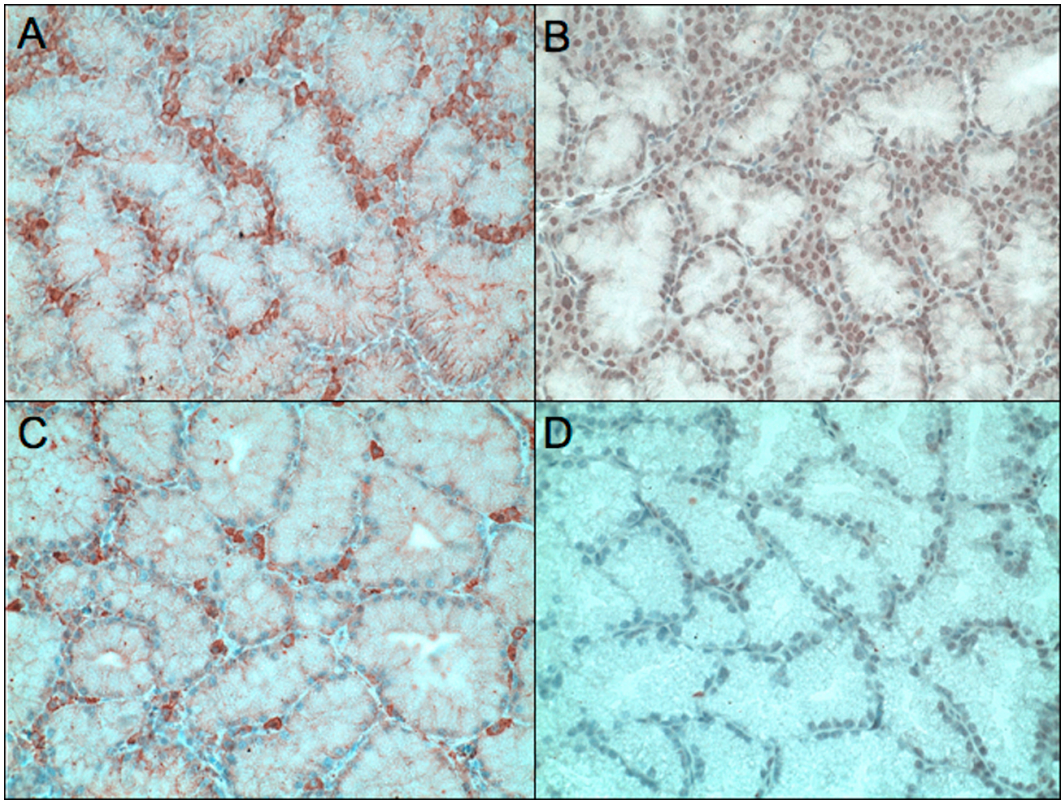

Figure 5.6. (A and B) Immunoperoxidase staining of IgA+ plasma cells in the American goldfinch Harderian gland using rabbit anti-house finch IgA sera (A) or pre-immunization control sera (B). (C and D) Immunoperoxidase staining of IgA+ plasma cells in the house sparrow Harderian gland using rabbit anti-house finch IgA sera (C) or pre-immunization control sera (D). Magnification 400×.

M. gallisepticum-specific IgA in house finch lachrymal samples

The anti-sera were also utilized in ELISA to detect *M. gallisepticum*-specific IgA in house finch lachrymal samples (Figure 5.7). After experimental inoculation, *M. gallisepticum*-specific IgA was detected at 2, 3, and 4 weeks post-infection in the lachrymal fluid (one-way ANOVA, $F(4,114) = 7.508$, $p < 0.0001$). Although the changes in optical density (OD) at 2, 3 and 4 weeks post-infection are relatively small, the mean background OD values at 0 and 1 week post-infection show little variation. After inoculating chickens with infectious bronchitis virus, Toro et al. (1994) also found low positive OD values for IgA in tears (Toro et al., 1994).

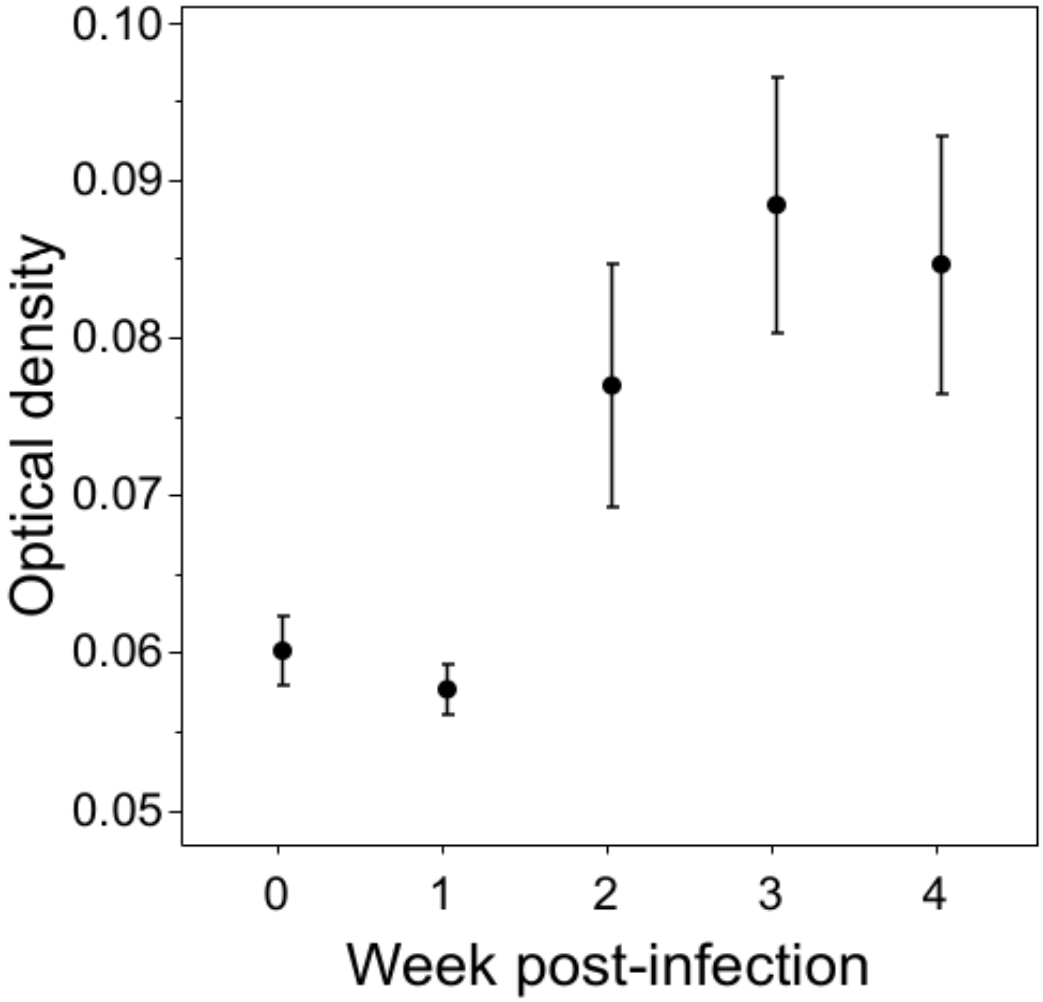

Figure 5.7. Detection of *M. gallisepticum*-specific IgA in lachrymal fluid. House finches were inoculated ocularly with *M. gallisepticum*. Lachrymal samples were taken pre-infection and weekly post-infection and used in ELISA to measure *M. gallisepticum*-specific IgA.

Although only small amounts (~0.75 μL) of lachrymal fluid were collected from the house finches, this was adequate to detect changes in IgA level as infection progressed. While providing a limited amount of sample, this collection method was simple, quick to perform, and posed little harm to the delicate conjunctival tissues.

In summary, this paper reports the first partial cloning of a passerine IgA constant region sequence and the first reagent developed specifically to detect IgA from a passerine species. The reagent has been applied successfully in Western blot, immunohistochemistry, and ELISA. The anti-sera will be used to study the mucosal antibody response of house finches infected with the bacterium *M. gallisepticum*.

Acknowledgement

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CHAPTER 6

Pathogenicity and immunogenicity of three *Mycoplasma gallisepticum* isolates in house finches (*Carpodacus mexicanus*)*

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6.1 Abstract

Mycoplasma gallisepticum (MG) has become a common cause of conjunctivitis in free-living house finches (*Carpodacus mexicanus*) since its emergence in the early 1990s. To date, temporal and spatial genotypic variation in MG has been documented, but phenotypic variation in pathogenicity and immunogenicity has not been examined. House finches were inoculated with MG isolates Virginia (VA)1994, California (CA)2006, or North Carolina (NC)2006, which were cultured from free-living house finches with conjunctivitis in 1994, 2006, and 2006, respectively. Infection with NC2006 resulted in the most severe eye lesions, highest pathogen loads, and highest levels of pathogen-specific lachrymal and serum antibodies. Infection with CA2006 caused the least severe eye lesions, lowest pathogen load, and lowest levels of antibodies. A small number of birds in each group developed protracted, severe disease in spite of robust antibody responses, suggesting that immunopathology may contribute to the lesions. Immunoblot analyses indicated that isolates are antigenically similar; thus, there may be partial cross-protection if a house finch encounters two or more strains of MG throughout the course of its lifetime. This study provides evidence that MG strains or strain variants circulating in house finch populations vary in their ability to cause disease, induce antibody responses, and persist in the host.

6.2 Introduction

In 1994, *Mycoplasma gallisepticum* (MG), a common respiratory pathogen of poultry, emerged as a cause of conjunctivitis in free-living house finches (*Carpodacus mexicanus*) (Ley et al. 1996, Dhondt et al. 1998) and rapidly resulted in major population declines (Hochachka and Dhondt 2000). Among house finches, MG is most likely transmitted via direct contact (Ley 2003) and fomites such as birdfeeders (Dhondt et al. 2007a). The organism can be cultured from conjunctival swabs of free-living house finches (Ley et al. 1996, Hartup et al. 2000) and experimental infection by conjunctival exposure results in the clinical signs seen under natural conditions (Kollias et al. 2004, Sydenstricker et al. 2006). Experimentally infected house finches develop partial immunity after initial infection. These birds remain susceptible to re-infection, but clinical signs are generally milder and of shorter duration (Sydenstricker et al. 2005).

Strains of MG isolated from commercial poultry are quite variable with respect to pathogenicity, transmissibility, and immunogenicity in chickens and turkeys (Rodriguez and Kleven 1980, Soeripto et al. 1989a, Soeripto et al. 1989b, Dingfelder et al. 1991, Sanei et al. 2007). While some variations at the genomic level have been documented among MG isolates from house finches (Pillai et al. 2003, Cherry et al. 2006), there has only been one recent report suggesting differences in pathogenicity based on experimental infections under controlled conditions (Hawley et al. 2010). Earlier studies using MG isolates collected from house finches in Virginia in 1994 (Sydenstricker et al. 2006), Georgia in 1995 (Farmer et al. 2002), and Alabama in 1999 (Farmer et al. 2005) produced clinical signs in experimentally infected birds, but variations in disease severity or immune responses were not documented among the isolates.

Variations in lesion severity and duration in house finches occur among individuals experimentally challenged with a given MG isolate. After experimental infection, eye lesions

resolve in most birds over the course of several weeks; however, often a small number of birds experience a severe, protracted form of the disease (Roberts et al. 2001, Sydenstricker et al. 2006). Development of chronic disease could be caused by co-infection with viruses (Adler et al. 1962) or other bacterial species such as *Escherichia coli* (reviewed by Kleven 1998a, Bradbury 2005), invasion of non-phagocytic cells (Winner et al. 2000), compromised immunocompetence (Cartner et al. 1998), or as a consequence of immunopathology (Bodhankar et al. 2010). The development of chronic lesions could also be due to the ability of MG to undergo immune evasion by antigenic variation of cell surface lipoproteins, most notably in the VlhA family of proteins. The multi-gene family encoding variants of VlhA comprises approximately ten percent of the MG genome (Papazisi et al. 2003). The main function of this gene family appears to be evasion of host antibody responses via phenotypic variation of the surface protein (Glew et al. 2000).

To characterize and compare the pathogenesis of MG infection in house finches, we challenged birds with MG isolates VA1994, CA2006, or NC2006 by the ocular route; these isolates were cultured from the conjunctivae of free-living house finches with eye lesions typical of MG infection in Virginia during 1994, California during 2006, and North Carolina during 2006, respectively. Eye lesion scores, pathogen loads, and lachrymal and systemic antibody responses were measured weekly post-infection. MG load was measured by quantitative (q)PCR assay targeting the *mcg2* gene, which is present as one copy per MG genome, while MG-specific serum antibodies and lachrymal IgA antibodies were measured by ELISA. Additionally, antigenic profiles of the three isolates were examined using immunoblotting.

6.3 Materials and methods

House finches

During August through October 2007, twenty-two house finches were captured via mist-nets and cage traps in Montgomery Co., VA (n=17), and Tompkins Co., NY (n=5) under permits from the Virginia Department of Game and Inland Fisheries (#031626), New York Department of Environmental Conservation (#99-039) and USGS Bird Banding Laboratory (#23513). In addition, required permits were obtained for house finch capture in California (SC-9517), transfer (USFWS MB158404-0), and importation to Virginia (VDGIF Exotic Import Permit 031729). Twenty house finches were captured in Contra Costa County, CA using mist nets and transported by commercial air to Virginia Tech in January 2008. One month prior to experimental challenge, all birds were transferred by state vehicle to Ithaca, NY under Cornell University IACUC protocol 2006-094. All birds were housed in individual cages in the Poultry Virus Isolation Facility at constant day length (12L:12D) and temperature and provided at *ad libitum* with drinking water and pelleted diet (Daily Maintenance Diet, Roudybush Inc., Woodland, CA).

During August through October 2008, thirty birds were captured in Tompkins Co., NY using mist nets under NY Department of Environmental Conservation (#99-039) and USGS Bird Banding Lab (#23513) permits and housed individually until transport to Virginia Tech via state vehicle under Virginia Tech IACUC protocol BIOL-07-052. Prior to use in the experiment, house finches were tested for the absence of antibodies to *Mycoplasma gallisepticum* (MG) by rapid plate agglutination (RPA) (Kleven 1998b). House finches in experiment 2 were also tested for the absence of infection by quantitative PCR assay. All procedures for animal care and use were approved by Virginia Tech and Cornell University IACUCs.

Mycoplasma gallisepticum isolates

MG isolate VA1994 (accession 7994-1, Avian Diseases Research Laboratory [ADRL], North Carolina State University College of Veterinary Medicine [NCSU CVM]) was obtained from a Virginia house finch with conjunctivitis in June 1994 (Ley et al. 1996). An expanded seventh passage culture (7994-1 7P, 5/24/04) was used for challenge and had a viable count of 1.66×10^7 color changing units (CCU)/ml determined by the most probable number method (Meynell and Meynell 1970). CA2006 (accession 2006.052-5, ADRL NCSU CVM) was acquired in June 2006 from a house finch with conjunctivitis in California. Cultures of this isolate were expanded using Frey's medium with 15% swine serum (modified from Kleven 1998b). The fourth passage culture (2006.052-5 4P, 11/12/07) was used for experimental infection and had a viable count of 3.37×10^6 CCU/ ml. The isolate NC2006 (accession 2006.080-5, ADRL NCSU CVM) was acquired in 2006 from a house finch with conjunctivitis in North Carolina. The fourth passage culture of NC2006 (2006.080-5 4P, 1/9/09) had a viable count of 3.04×10^8 CCU/ml. All MG stocks were stored at -70°C until immediately prior to use. Upon thawing, VA1994 was diluted 1:5 in Frey's media to approximate the dose (CCU/ml) of CA2006 in experiment 1, while NC2006 was diluted 1:18 in Frey's media prior to use in experiment 2.

Challenge studies

Experimental design. Two experiments were conducted (see Table 6.1 for a summary of experimental designs). In experiment 1, the pathogenesis of infection was examined for the VA1994 and CA2006 isolates. Two groups of twenty single-housed house finches were challenged by applying 0.05 ml containing approximately 3.3×10^6 CCU/ml of VA1994 or

CA2006 to both palpebral conjunctivae of each bird. Four birds served as negative controls and received 0.05 ml of media alone in each conjunctiva. Two birds inoculated with CA2006 had positive RPA test results prior to experimental infection; the two birds did not have clinical signs of infection and were negative for the MG qPCR assay. These results may have been false positives or may represent prior exposure to MG. These two birds were excluded from statistical analyses.

The experimental design of experiment 1 is also described by Hawley et al. (2010); the study found evidence that, on average, host population origin and genetic diversity did not have an effect on host resistance to infection, as measured by lesion score and pathogen load.

In experiment 2, the pathogenesis of infection was compared for the VA1994 and NC2006 isolates. Thirteen single-housed house finches were challenged as described for experiment 1 with 0.05 ml containing 1.7 $x10^7$ CCU/ml of VA1994 or NC2006. Four birds served as negative controls and received 0.05 ml of media in each conjunctiva.

Table 6.1: Experimental design of challenge studies using isolates VA1994, CA2006, and NC2006. In two experiments, house finches were infected with three isolates of *Mycoplasma gallisepticum* (MG). MG inocula were quantified using color changing units (ccu) and were administered to the right and left conjunctivae of each bird.

Experiment	MG isolate or control	Sample size (house finches)	Approximate dose (total ccu per eye)
1	VA1994	20	1.7×10^5
1	CA2006	20	1.7×10^5
1	controls	4	0
2	VA1994	13	8.3×10^5
2	NC2006	13	8.3×10^5
2	controls	4	0

Lesion scoring. Birds were examined for eye lesions at day 2, day 7, and weekly thereafter. Lesions were scored on a 0 to 3 scale (Sydenstricker et al. 2005) as follows: 1= minor swelling around the eye ring, 2= moderate swelling and eversion of the conjunctival tissue, and 3= the eye is nearly hidden by swelling and crusted exudates.

*Sample collection***.** Lachrymal fluids were collected pre-challenge and weekly until eight weeks post-challenge using modified Schirmer tear test strips (Schering-Plough Animal Health, Union, NJ) as described by Grodio et al. (2009). After collection, strips were placed in microcentrifuge tubes containing 250 µl of PBS/0.05% Tween 20 (PBS-T); tubes were vortexed vigorously, tear strips were discarded and samples were stored at -20°C until assayed.

Conjunctival swabs for qPCR assays were collected before challenge, on day 2, day 7 and

weekly thereafter. Sterile forceps (Fisher Scientific, Pittsburgh, PA) were used to hold the lower palpebra and a cotton-tipped wooden-handle swab (Fisher Scientific) dipped in tryptose phosphate broth (TPB) was inserted into the lower palpebral sac. Immediately after sample collection, the tips of the swabs were placed into 300 μl of sterile TPB. Swabs were swirled and wrung out on the inside of tubes to remove liquid from the swabs before discarding. Samples were frozen at -20°C prior to DNA extraction.

Blood was collected from the wing vein into one to two lithium-heparinized microcapillary tubes. Tubes were spun in a tabletop centrifuge and plasma was collected and frozen at -20°C.

Quantification of pathogen load

MG loads were quantified using the qPCR assay as described by Grodio et al. (2008). Genomic DNA was extracted from conjunctival swab samples using Qiagen DNeasy 96 Blood and Tissue kits (Qiagen, Valencia, CA). Primers (Forward: 5'-GGTCCTAATCCCCAACAAAGAAT, Reverse: 5'-CTTGGTTGGTTCATATTAGGCATTT, Integrated DNA Technologies, Coralville, Iowa) and probe (5'-6-FAM-CCACAGGGCTTTGGTGGCCCA-BHQ-1, Integrated DNA Technologies) targeting the *mgc2* gene of MG were used. Each 25 μl reaction contained 12.5 μl TaqMan PCR Universal Master Mix (2x), 0.125 μl each of 100 μM forward and reverse primers, 0.25 μl of 10 μM probe, 7 μl DNase-free water, and 5 μl of DNA sample. Cycling was performed using an ABI Prism 7700 (Applied Biosystems, Foster City, CA) and the following parameters: 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 sec and 60°C for 60 sec. Standard curves were generated for each run using 10-fold serial dilutions of plasmid DNA containing 2.53×10^1 to 2.53×10^8 copy numbers of a 303 bp *mgc2* insert. The average copy

numbers for each bird was calculated using the values for the right and left eye at each time point and $\log_{10}$ transformed for statistical analysis. We eliminated a single qPCR value from analysis that was obtained from one eye of a bird *prior to inoculation*, as this value (11.34 $\log_{10}$) was much higher than the maximum value of MG (3.08 $\log_{10}$) detected in any individual post-inoculation in experiment 1 (Hawley et al. 2010).

Antibody assays

Serum antibodies. The Idexx FlockChek *Mycoplasma gallisepticum* antibody ELISA kit (Idexx, Westbrook, ME) was used to measure serum antibodies with modifications to the manufacturer's protocol. All steps were carried out at room temperature. A blocking step included the addition of 300 µl of 1% BSA (Pierce 10X BSA, Thermo Fisher Scientific, Rockford, IL) in PBS for forty minutes followed by a washing step. All washing steps consisted of washing the plate three times with 350 µl of PBS with 0.05% Tween 20 (PBS-T) using ELx50 Auto Strip Washer (Bio-Tek, Winooski, VT). House finch serum samples were diluted 1:50 in the kit's sample buffer, 100 µl were added to duplicate wells and incubated for 1 h. Plates were then washed, 100 µl pre-diluted kit antibody (goat anti-chicken/goat anti-turkey, HRPO conjugate) was added to the wells and incubated for 1 h, followed by a washing step. TMB substrate and stop solution were added as per manufacturer's protocol and absorbance was read at 630 nm using an ELx800 Universal Microplate Reader (Bio-Tek). The average value was calculated for each sample for further analysis. To control for inter-assay variation, the ELISA values were calculated using the equation (sample mean – negative control)/(positive control – negative control).

Lachrymal antibodies. MG-specific IgA antibodies in the lachrymal fluids were measured using the Idexx FlockChek *Mycoplasma gallisepticum* antibody ELISA kit as described previously (Grodio et al. 2009). All steps were carried out at room temperature. First, 300 µl of 1% BSA in PBS-T was added to each well and incubated for forty minutes. After washing three times with 350 µl of PBS-T, lachrymal samples (0.3 µl lachrymal fluid in 100 µl PBS-T) were added to the wells and incubated for 1 h. After washing with PBS-T, rabbit anti-house finch IgA heavy chain serum (diluted 1:200 in PBS-T) was added to each well and the plates were incubated for 1 h. After washing, goat anti-rabbit IgG (Bethyl Laboratories, Montgomery, TX) diluted 1:60,000 in PBS-T was added and the plates were incubated for forty minutes. After washing, TMB substrate and stop solution were added as per manufacturer's protocol and absorbance was read at 630 nm. To control for inter-assay variation, the ELISA values were calculated as described above.

Immunoblots

Broth cultures of VA1994, CA2006, and NC2006 described above were spun down at ~18,000g at 4°C for thirty minutes. The cells were washed three times with sterile PBS and after centrifugation, supernatant was removed and pellets were placed on ice. Washed cells were solubilized in 20 mM Tris containing 0.1% SDS, 140 mM NaCl, 1% IGEPAL, 10% glycerol, 0.5% deoxycholate acid, and 2 mM EDTA. Protein concentration was determined using Bradford's assay and a BSA standard curve (New England Biolabs, Ipswich, MA). Solubilized proteins of the MG isolates (4 µg/well) were diluted 1:1 with sample buffer (4% SDS, 20% glycerol, 50 mM Tris, pH 6.8, 0.1% bromophenol blue, 10% 2-mercaptoethanol), incubated at 100°C for 10 minutes, and separated on a SDS-PAGE gel consisting of 5% stacking/10% resolving gels. Proteins were transferred to 0.45 µm nitrocellulose membranes (Bio-Rad,

Hercules, CA). Membranes were incubated in blocking buffer (Tris-buffered saline with 1% Tween 20 [TBS-T] plus 5% non-fat dry milk [Nestle, Vevey, Switzerland]) for 2 h at room temperature. After washing in TBS-T, individual lanes were cut from the blot and incubated with sera of infected house finches (14 days post-infection) diluted 1:200 in blocking buffer. The blots were incubated overnight at 4ºC, washed with TBS-T and incubated with goat anti-bird IgG-HRP (Bethyl Laboratories, Montgomery, TX), diluted 1:60,000 in blocking buffer for 1 h at room temperature. Blots were washed and SuperSignal WestPico Chemiluminescence substrate (Pierce, Thermo Fisher Scientific) was applied as recommended by the manufacturer. The blots were covered with plastic wrap and exposed to film (Amersham Hyperfilm ECL, GE Healthcare, Piscataway, NJ) in a dark room.

Statistical analyses

The longitudinal nature of the lesion, qPCR, and antibody data necessitated the use of a general linear mixed model to account for repeated measures on individual birds. Past experience (Hawley et al. 2010) suggested that both an individual bird random effect and temporally auto-correlated errors were statistically important for model fit, thus we fit models that contained these properties. We examined the influence of experiment (challenge 1 versus challenge 2), MG isolate, day post-inoculation (PI), and all interactions on eye score, pathogen load, and serum and lachrymal antibody responses using a general linear mixed model (Pinheiro and Bates 2000) that included a random intercept effect for individual birds and errors within a bird that were autocorrelated across day PI (corCAR1 in R package nlme, R Core Development Team 2006, Pinheiro et al. 2007). Separate models were fit to determine the repeatability of the two challenge

experiments with VA1994 and the effects of MG isolate, days PI, and the interaction across all challenge studies.

To quantify pathogen clearance and disease recovery, we calculated survival curves based on the presence and absence of pathogen (qPCR) and lesions. Because these were only observed weekly, the exact time of clearance or disease recovery is only known to the weekly interval. Thus, we used techniques appropriate for this interval-censored data to construct non-parametric survival curves and test for differences between MG isolates (icfit and ictest, respectively, in the R package interval, Fay and Shaw 2010). We fit separate curves for each MG isolate for both pathogen load and lesion recovery and then tested for differences between strains of the survival curves using a logrank test (Fay and Shaw 2010). For this analysis, we pooled data across experiments for VA1994 because of the very small sample size for estimating survival curves.

In order to explore the relationship between pathogen load (qPCR), virulence (eye lesion), and serum and lachrymal antibody responses across individual finches and pathogen strains, we summed each response for each bird over the first 63 days PI. We then calculated rank correlations between the responses and used multivariate analysis of variance (MANOVA) to explore the effects of MG isolate on the multivariate response. We used a randomization test to validate the results from the MANOVA because assumptions of multivariate normality and equality of within-group covariance were likely not strictly met.

GraphPad PRISM (version 5.0b, GraphPad Software, Inc., San Diego, CA) was used to generate the graphs illustrated in Figure 6.5.

6.3 Results

Repeatability of challenge studies using VA1994

Birds were inoculated with VA1994 in two consecutive years, with a higher dose of inoculum used in experiment 2. Antibody responses to the VA1994 isolate showed similar trends in both experiments, but experiment 2 produced slightly higher pathogen load and lesion score and these differences were significantly different between experiments (Figure 6.1). For lesion score, the average effect for experiment ($F_{1,31} = 1.15$, $p = 0.29$) was not significant but there was a significant interaction between days PI and experiment ($F_{9,279} = 1.98$, $p = 0.04$), such that lesion score had a higher peak at weeks 1-4 PI in experiment 2 (Figure 6.1A). For MG load, both the average effect ($F_{1,31} = 36.26$, $p < 0.0001$) and days PI by experiment interaction effect ($F_{8,248} = 5.18$, $p < 0.0001$) was significant and showed that qPCR was higher across all days PI in experiment 2 (Figure 6.1B). The serum antibody response was not significantly different between experiments ($F_{1,31} = 0.66$, $p = 0.42$) or for the interaction between experiment and days PI ($F_{8,242} = 0.75$, $p = 0.65$; Figure 6.2A). For lachrymal IgA, the effect of experiment was not significant ($F_{1,31} = 0.26$, $p = 0.62$) but the interaction between days PI and experiment was significant ($F_{7,216} = 3.49$, $p = 0.002$). However, inspection of the estimated effects showed that there was no consistent difference between experiments, as the estimated interaction effect between days PI and experiment for IgA seemed to fluctuate between negative and positive each week (Figure 6.2B). Because of these obvious differences between experiments in lesion scores, qPCR, and antibody response, data from birds infected with VA1994 were not pooled in subsequent analysis.

Lesion scores

When all isolates were analyzed together, all estimated effects of isolate, days PI, and the isolate by days PI interaction were significant ($p < 0.0003$). Birds infected with NC2006 had the highest lesion scores, while birds infected with CA2006 had the lowest lesion scores (Figure 6.1A). Birds infected with VA1994 in either experiment had intermediate scores. In experiment 2, one uninfected control bird was noted to have irritation of the right eye at day 7 but no clinical signs were noted thereafter. This bird subsequently was qPCR positive and displayed elevated serum antibodies and lachrymal IgA at various time points.

Pathogen load

Quantitative PCR data showed significant effects for isolate, days PI, and the interaction between days PI and isolate ($p < 0.0001$). Birds infected with NC2006 had the highest MG load compared to the other isolates and infection with CA2006 or VA1994 in experiment 1 resulted in lowest bacterial loads (Figure 6.1B), perhaps due to a dose effect (Table 6.1). Pathogen load early in the infection (day 2 PI) was very different across isolates (Figure 6.1B). Birds infected with VA1994 in either experiment had the lowest bacterial loads at day 2 PI, birds infected with CA2006 had intermediate bacterial loads at day 2 PI, while birds infected with NC2006 had the highest bacterial load on day 2 PI. At one week PI, bacterial load appeared to increase for all isolates other than CA2006. After one week PI, bacterial load appeared to decrease, except for NC2006, which maintained high bacterial load until week 5 PI.

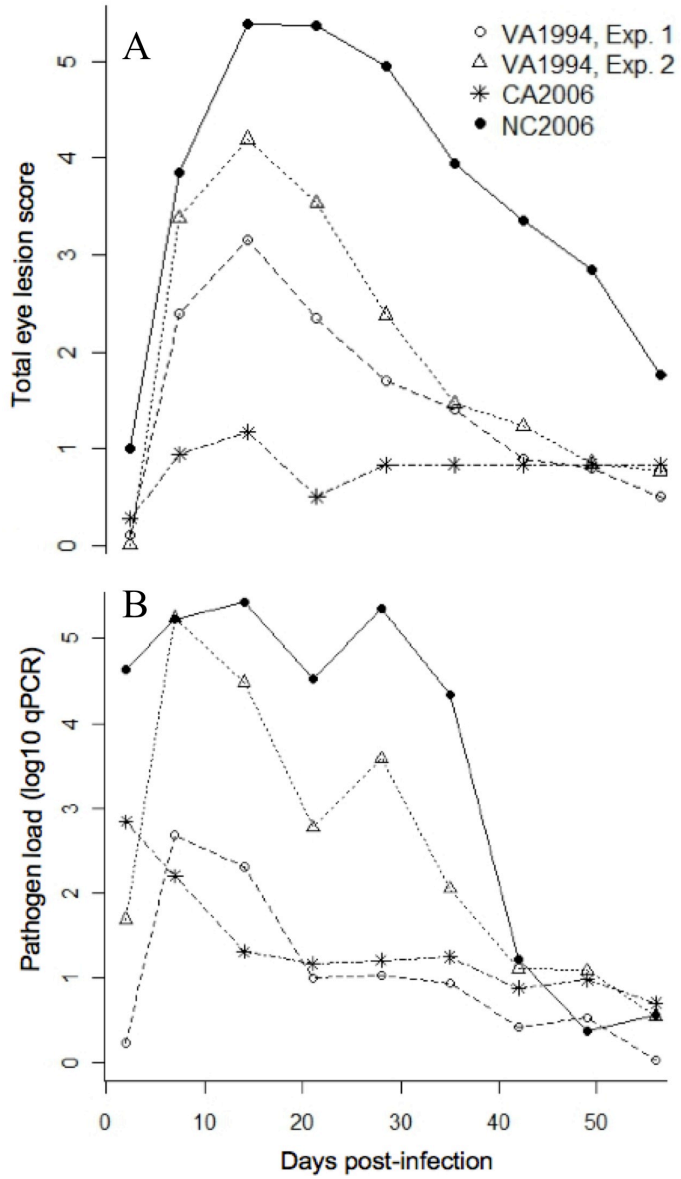

Figure 6.1. Lesions and pathogen load in house finches after infection with one of three isolates of *Mycoplasma gallisepticum* (VA1994, CA2006, or NC2006). Conjunctival lesions (A) were graded on a scale from 0-3 and averaged bilaterally. Pathogen load in the conjunctiva was quantified for eight weeks post-infection (B). Pathogen load is approximated by *M. gallisepticum mgc2* gene copy number, determined by quantitative PCR.

Antibody responses

Serum and lachrymal antibody responses showed similar patterns. All estimated effects (isolate, days PI, and the interaction between days PI and isolate) in the model were statistically significant ($p < 0.001$) for systemic antibody response; for local antibody response, all effects (days PI, and the interaction between days PI and isolate) were significant ($p < 0.0001$) except for the main effect of isolate ($p = 0.37$). Inspecting the estimated effects (Figure 6.2) shows that NC2006 elicited greater antibody responses after day 21 PI than VA1994 or CA2006 both systemically (Figure 6.2A) and locally in the lachrymal fluid (Figure 6.2B). Serum antibody response to VA1994 was higher than CA2006 at weeks 2, 3, and 4 post-infection (Figure 6.2A).

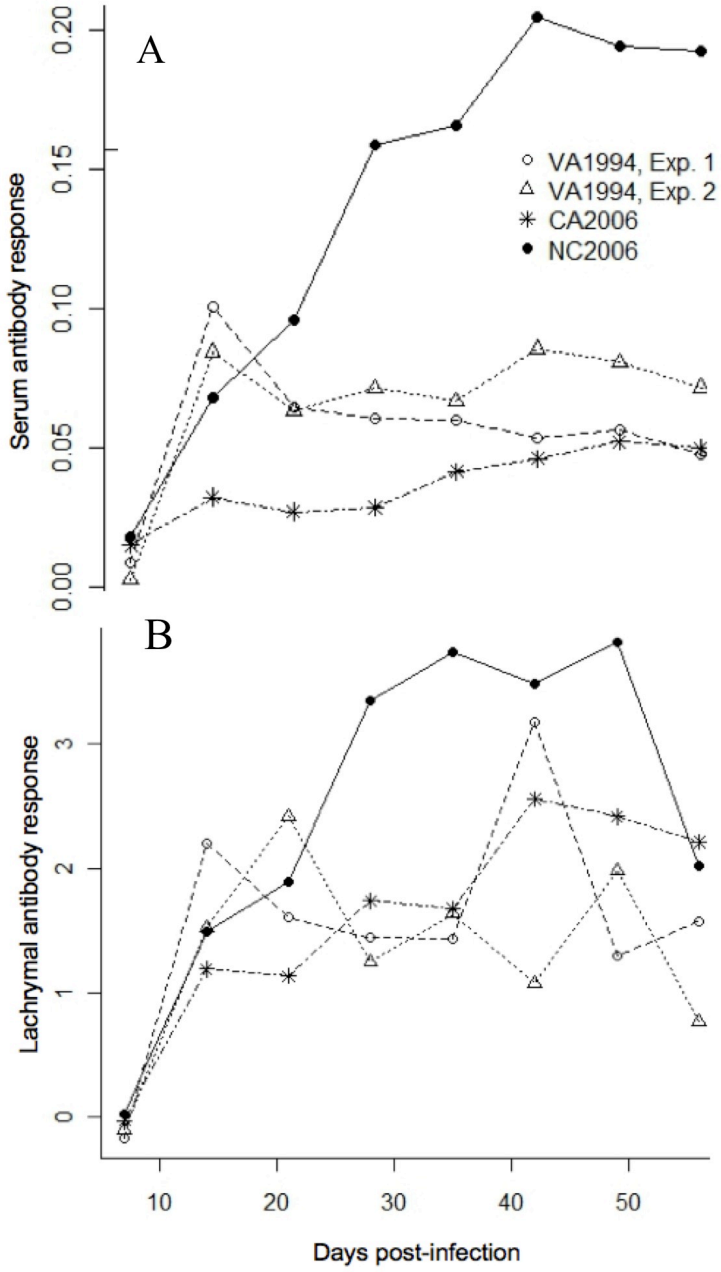

Figure 6.2. *Mycoplasma gallisepticum*-specific antibodies produced after inoculation of house finches with one of three *M. gallisepticum* isolates (VA1994, CA2006, or NC2006). Serum antibodies (A) and lachrymal IgA (B) were measured weekly post-infection.

Pathogen clearance and disease resolution

The time to disease resolution varied with MG isolate (Logrank test, $\chi^2 = 8.43$, $p = 0.01$; Figure 3A). Birds infected with CA2006 recovered from lesions fastest, those infected with NC2006 recovered slowest, and birds infected with VA1994 recovered at an intermediate rate.

The observed pattern of pathogen clearance was similar to lesion recovery in that VA1994 was intermediate and CA2006 was fastest, but this was not statistically significant (Logrank test, $\chi^2 = 0.27$, $p = 0.88$; Figure 6.3B). Given these results, it appears that birds infected with CA2006 recover from lesions much faster than they clear pathogen (compare Figure 6.3A to Figure 6.3B).

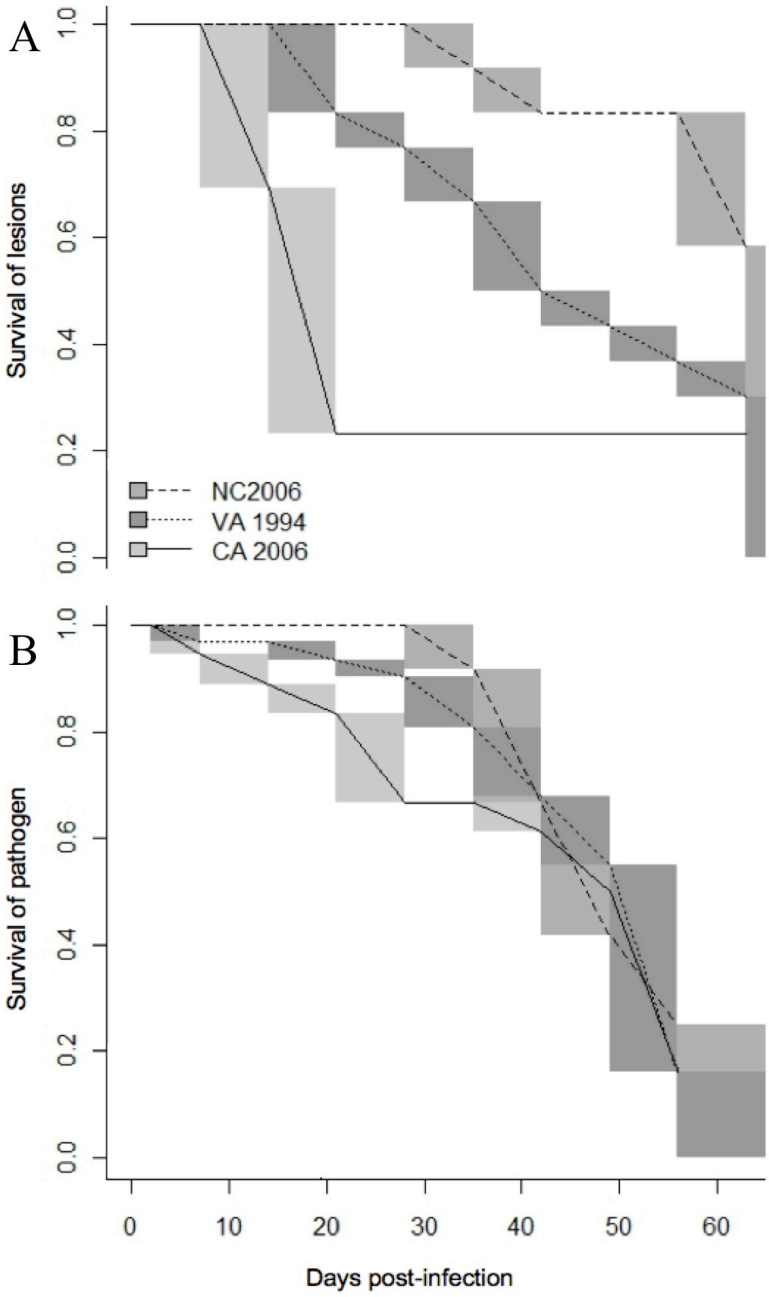

Figure 6.3. Survival curves for (A) lesions and (B) pathogen load based on non-parametric maximum likelihood techniques for interval-censored data. Shaded box are "Turbull intervals" where the survival function was determined to be changing but the exact functional form of the change is unknown, given the observed data. Dark shading (dotted line) is VA1994, medium shading (dashed line) is NC2006, and light shading (solid line) is CA2006.

Pathogen load and antibody response compared to disease severity

All pair-wise correlations between lesion score, pathogen load, serum antibody, and lachrymal IgA antibody response were highly significant (Kendall's Tau > 0.35, $p << 0.001$; Figure 6.4). Pathogen strain or experiments differed in the multivariate response of between lesion score, pathogen load, serum antibody, and lachrymal IgA antibody (MANOVA: $F_{12, 167} = 9.22$, $p < 0.001$). Inspection of the pair-wise relationship between pathogen load and eye lesion score suggests that the isolate (or experiments) differed in this response (Figure 6.4A); whereas, the relationship between antibody response and pathogen load, eye lesion score, or other pair-wise relations did not seem to discriminate between isolates (Figure 6.4B, other pair-wise relations not shown). This difference between isolates shows that NC2006 and VA1994 in experiment 2 had relatively high pathogen loads and variable eye lesion scores across birds (Figure 6.4A). The isolates CA2006 and VA1994 from experiment 1 produced lower pathogen load scores and more variable eye lesion scores. This indicates that the main difference between isolates is their propensity to cause high pathogen load or in the dose of inoculum administered.

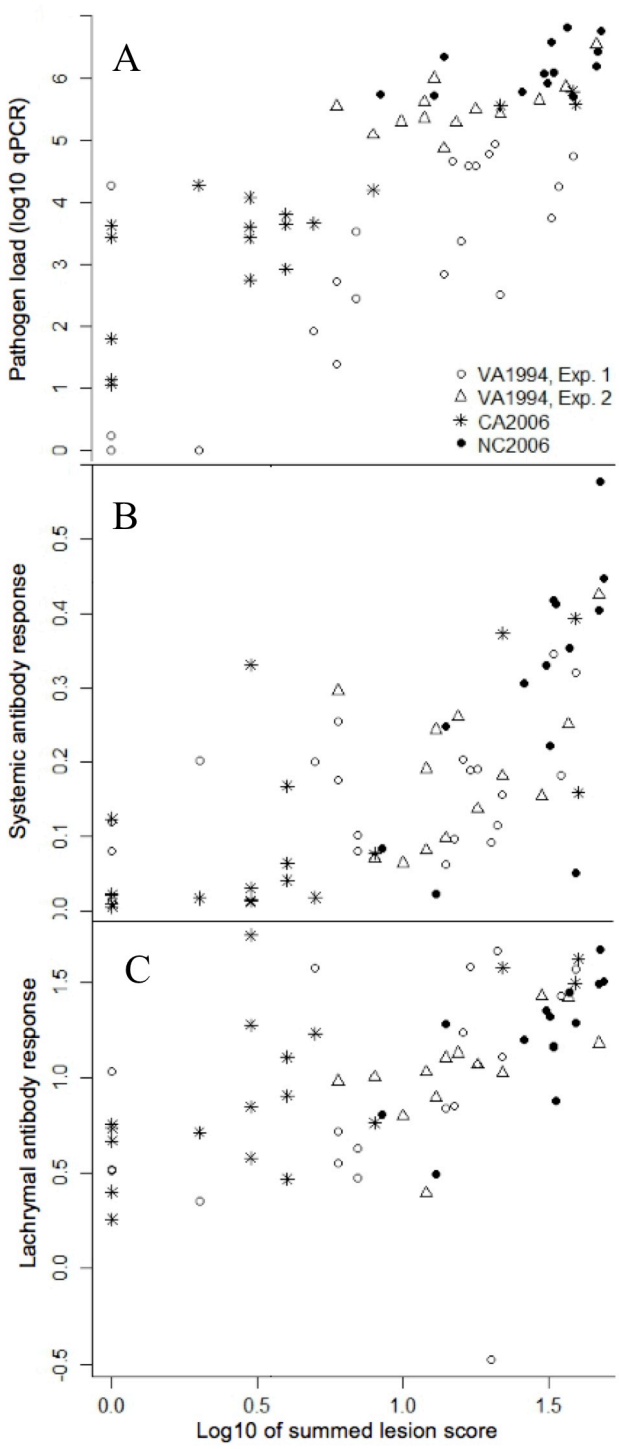

Figure 6.4. Relationships between summed eye lesion score and pathogen load (A), serum antibodies (B), and lachrymal IgA (C).

Cases of chronic disease

To determine if secondary bacterial infections were present in birds exhibiting severe lesions at the end of the experiment, conjunctival swab samples from three birds infected with CA2006 were placed in 200 μL of TPB. Samples were submitted to the NYS Animal Health Diagnostic Center at Cornell University for aerobic bacterial culture. No bacterial species were cultured from the conjunctival swabs.

These three birds displayed such marked differences in lesion scores compared to the rest of the birds infected with CA2006 that the qPCR and antibody results are shown separate for this small group in Figure 6.5. These birds had greater levels of MG load and produced higher levels of serum antibodies and lachrymal IgA compared to the rest of the birds which developed a mild, shorter form of the disease.

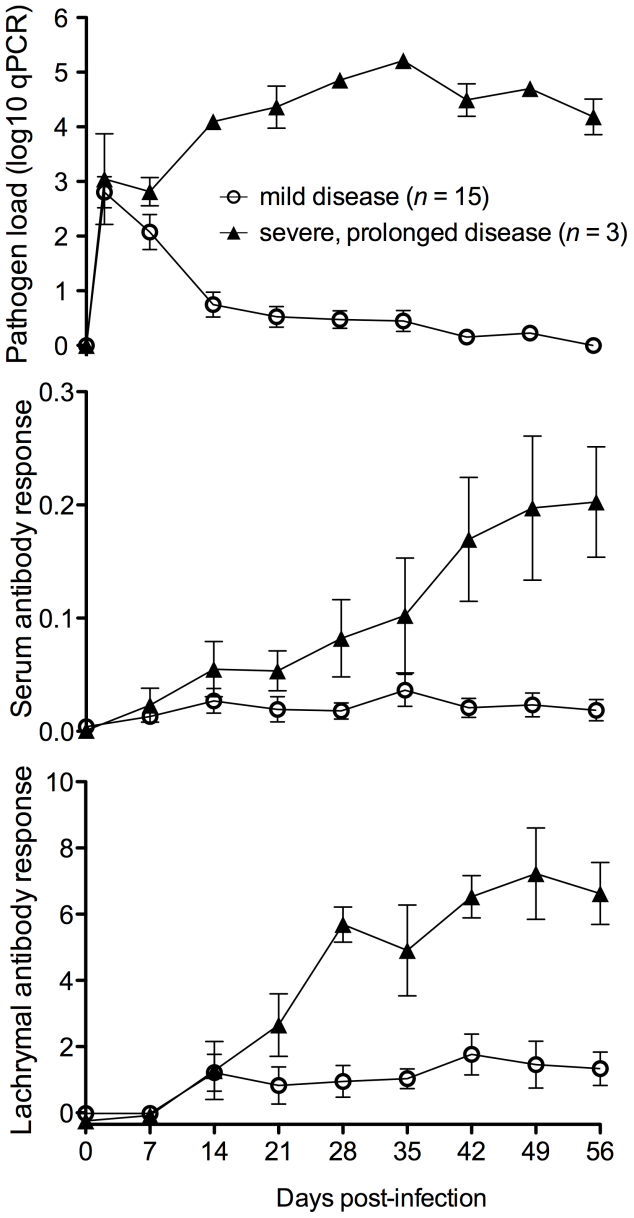

Figure 6.5. Pathogen load, serum antibody response, and lachrymal IgA antibody response in birds infected with CA2006, grouped by disease severity.

Immunoblots

Immunoblotting was performed by separating proteins of MG isolates on an SDS-PAGE gel and incubating the resulting blot with sera from birds infected with the homologous isolate (Figure 6.6). Sera from birds infected with NC2006 and VA1994 recognized approximately eight to ten proteins at two weeks post-infection. Sera from birds infected with CA2006 reacted with fewer proteins. "Heterologous" immunoblotting was used to examine the potential for cross-immunity and was performed by separating proteins of a given isolate (e.g. VA1994) and incubating the blot with sera from a bird infected with a different isolate (CA2006 or NC2006). The heterologous immunoblots showed that antibodies directed against one isolate could also recognize proteins of the other isolates. Interestingly, sera from birds infected with NC2006 and VA1994 reacted to CA2006 antigen with greater intensity than CA2006-specific antisera reacted to CA2006 antigen.

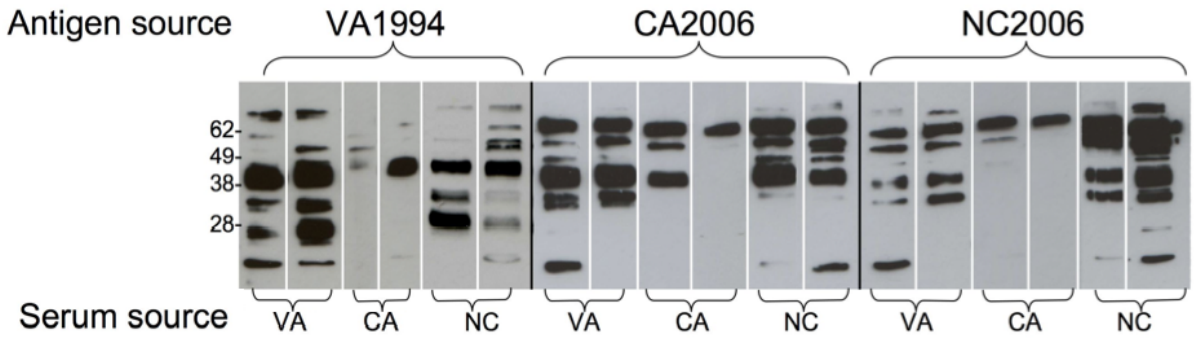

Figure 6.6. Immunoblots of *M. gallisepticum* proteins incubated with antisera collected from house finches at two weeks post-infection. "Antigen source" indicates which *M. gallisepticum* isolate was run on the SDS-PAGE gel. "Serum source" identifies antisera from birds infected with a specific isolate of *M. gallisepticum* (VA = VA1994, CA = CA2006, NC = NC2006). Approximate sizes in kDa are indicated on the left.

6.4 Discussion and conclusions

The results of this study demonstrate that isolates of MG from house finches with conjunctivitis can vary greatly in ability to cause disease and induce production of local and systemic antibodies. Infection with NC2006 resulted in the most severe eye lesions, highest MG load, and greatest levels of MG-specific lachrymal IgA and serum antibodies after day 21 PI. Infection with CA2006 caused the least severe eye lesions, lowest pathogen load, and lowest levels of antibodies in most individuals. Infection with VA1994 resulted in intermediate levels of lesion severity, pathogen load, and antibody response. NC2006 and CA2006 were collected from house finches with eye lesions during the same year but at different locations; therefore, it is likely that numerous strain variations of MG are circulating in free-living house finch populations and that they vary in their ability to cause disease and induce an antibody response.

The *mgc2* gene is present as a single copy in the MG genome, and we used *mgc2* copy number present in conjunctival swab samples to estimate pathogen load. If greater numbers of MG in the conjunctiva results in more transmission to susceptible individuals, our results suggest that isolates may differ in the potential for disease transmission, with NC2006 being the most transmissible of the isolates tested. In addition to MG load, transmissibility is likely dependent on many other factors, such as duration of infection, presence of fomites (Dhondt et al. 2007a), social status of the bird (Hawley et al. 2007b), house finch population density (Hochachka and Dhondt 2000), behavioral changes associated with infection (Hawley et al. 2007a, Bouwman and Hawley 2010), flock age composition (Altizer et al. 2004), and ambient temperature.

As Figure 6.4 demonstrates, serum and lachrymal antibody responses were positively correlated with lesion scores, suggesting that the presence of antibodies may contribute to the disease process or that antibodies may be ineffective at clearing MG. Mycoplasma species have

multiple adaptations that allow them to survive in the host despite a strong immune response directed against them (for review, see Simecka 2005). Mycoplasma species undergo variation of surface antigens *in vitro* (e.g., Zhang and Wise 2001) and *in vivo* (Levisohn et al. 1995), likely an adaptation to evade the host antibody response. The MG VlhA surface protein undergoes phenotypic variation *in vitro* in response to VlhA-specific monoclonal antibodies (Markham et al. 1998); additionally, *in vivo* variation of the VlhA protein has also been documented during MG infection in chickens (Glew et al. 2000). Another mechanism that allows Mycoplasma species to modulate the immune response includes the ability to induce polyclonal activation of lymphocytes (Stanbridge 1982, Williamson et al. 1986, Cole and Atkin 1991). The ability to non-specifically activate lymphocytes likely contributes to lesion severity (Naot et al. 1981).

During Mycoplasma infection, the adaptive immune response plays a role in clearance of the organism but also contributes to the clinical lesions observed. In chickens, antibody responses are important for clearing MG infection; bursectomized chickens show increased susceptibility to infection (Adler et al. 1973, Lam and Lin 1984). Additionally, vaccinated chickens develop milder lesions and have increased numbers of MG-specific IgG and IgA secreting plasma cells (Javed et al. 2005). Studies utilizing immunocompromised hosts provide evidence for both the importance of the adaptive immune response and for the immunopathology that ensues during Mycoplasma infection. SCID (severe combined immunodeficiency) mice infected with *Mycoplasma pulmonis* develop less severe lung lesions but have similar pathogen loads in the lungs compared to controls; SCID mice also had greater quantity of *M. pulmonis* in the spleen and lesions in other extrapulmonary sites when compared to controls (Cartner et al. 1998). T-cell deficit hamsters infected with *M. pneumoniae* had less severe lesions but had higher pathogen loads when compared to immunocompetent controls (Taylor et al. 1974).

Bodhankar et al. (2010) found that IFN-γ deficient mice immunized with *M. pulmonis* antigen and then infected with *M. pulmonis* developed more severe lesions than controls, and IL-4 deficient mice developed less severe disease than the IFN-γ deficit mice and had greater ability to clear infection; their study provides evidence of the importance of IFN-γ in protection and the contribution of IL-4 mediated responses to the disease pathology.

In concordance with past experiments (Kollias et al. 2004, Sydenstricker et al. 2006, Dhondt et al. 2007b, Hawley et al. 2010), this study demonstrates that there is considerable individual variation in response to infection; birds infected with the same MG isolate at identical doses can develop very different courses of disease. All isolates caused severe, protracted disease in a small number of individuals. Although on average infection with CA2006 resulted in a very mild, short-lasting conjunctivitis, three out of twenty (15%) finches experienced a prolonged and severe disease course. At the end of eight weeks, these birds had large numbers of MG in the conjunctiva; thus, it is likely that these individuals could serve as a source of infection for other susceptible birds. It is unclear whether or not the birds that develop severe, persistent infections play an important role in transmission of disease in free-living populations. Birds with severe lesions have reduced activity (Kollias et al. 2004) and likely compromised vision; these birds may not survive in the wild due to increased risk of predation and inability to forage for food. The availability of bird feeders, however, could contribute to their survival. Birds with conjunctival lesions tend to feed for longer intervals of time at feeders than those without clinical signs and tend to move less frequently between feeders and nearby perches (Hawley et al. 2007a). Altizer et al. (2004) found that free-living house finches with severe lesions had poorer measures of body condition; however, Hartup et al. (2000) reported no difference in body condition score between diseased and non-diseased birds. In the presence of a stable food source,

the individuals with severe, protracted disease may be able to persist in the wild, serving as a source of infection.

The three birds with persistent lesions infected with CA2006 had high numbers of MG in spite of developing a very robust antibody response. While the importance of antibody-mediated clearance of MG has been demonstrated for chickens (Javed et al. 2005, Gaunson et al. 2006), it is clear from the CA2006 experimental challenges that MG can persist in great numbers in the house finch host in the presence of vigorous serum and lachrymal antibody responses. The ability of MG to persist for prolonged periods in some individuals may be due to the ability of the bacteria to vary surface antigens targeted by the host antibody response, particularly the VlhA family of surface proteins (Levisohn et al. 1995, Gorton and Geary 1997, Markham et al. 1998); The reason protracted disease only occurs in a fraction of individuals in unclear, but may be due to genetic variation among house finches.

The ELISA and immunoblot results show that the majority of birds develop only a weak antibody response to CA2006 and react to fewer MG proteins compared to birds infected with VA1994 and NC2006 isolates. Birds infected with CA2006 had high pathogen load in the conjunctiva at day 2, but levels declined quickly thereafter. This may represent a decreased ability of CA2006 to colonize house finch conjunctiva (e.g., decreased ability to attach to mucosa, decreased growth rate, or increased clearance of bacteria by the innate immune system). The heterologous immunoblot results show that isolates are antigenically similar and suggest that cross-immunity may be possible; thus, a house finch infected with one strain variant of MG may have partial immunity when infected with a different circulating variant.

The three isolates used in these experiments were collected from free-living house finches with conjunctivitis. There are likely a variety of MG strains or strain variants circulating

in free-living house finch populations and our results suggest that they vary in their ability to persist in their host and in their ability to cause disease and induce an antibody response. Characterizing this phenotypic variation is critical for a broader understanding of the dynamics of MG in house finches, which continues to cause annual epidemics in free-living populations.

Acknowledgments

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CHAPTER 7

Chronic *Mycoplasma* conjunctivitis in house finches: host antibody response and *M. gallisepticum* VlhA expression*

*Submitted. Grodio, J.L., Ley, D.H., Schat, K.A. and D.M. Hawley. Chronic *Mycoplasma* conjunctivitis in house finches: host antibody response and *M. gallisepticum* VlhA expression.

7.1. Abstract

Previous studies have shown that house finch field isolates of *Mycoplasma gallisepticum* (MG) vary in virulence and ability to induce an antibody response. After experimental inoculation, MG causes persistent, severe disease in a subset of individuals. In this study, we further characterized MG infection using five field isolates, with an emphasis on chronically diseased birds. After experimental inoculation of house finches, MG load was measured by quantitative PCR and anti-MG antibody responses were measured by ELISAs. Birds with chronic disease had significantly higher pathogen loads and antibody responses than did birds without chronic disease. Using a monoclonal antibody (MAb86) specific for a variant of the MG VlhA adhesin and immunodominant surface protein, we show that VlhA expression differs among MG isolates in this study, and that *in vivo* VlhA changes occur in house finches infected with MG. Overall, our results suggest that chronic MG disease has both pathogen-mediated and immunopathologic components.

7.2. Introduction

Mycoplasma gallisepticum (MG) is a common cause of acute and chronic respiratory disease in chickens and infectious sinusitis in turkeys (Ley 2003). In 1994, MG was documented in a novel host, the house finch (*Haemorhous*, formerly *Carpodacus*, *mexicanus*) (Ley et al. 1996). In this host, MG causes predominantly conjunctivitis, with respiratory signs being much less common compared to infection in poultry (Ley et al. 1996, Ley, 2003). Free-living house finch populations suffered major declines after the emergence of MG (Hochachka and Dhondt 2000), and the disease has now spread throughout the North American range (Ley et al. 2006).

When challenged with identical doses of a given MG isolate, infected house finches exhibit variation in disease progression, such as onset and severity of conjunctivitis, and duration of clinical disease (Kollias et al. 2004, Sydenstricker et al. 2005). Roberts et al. (2001) found that 73% of infected house finches developed acute, self-limiting conjunctivitis while 27% suffered from chronic disease and had a higher mortality rate. Grodio et al. (2012) found that a small percentage of house finches infected with an MG isolate of relatively low virulence developed severe, prolonged disease characterized by high conjunctival MG load and high levels of MG-specific tear IgA antibodies and serum antibodies.

Mycoplasma species are known for their ability to cause chronic infections and diseases, which has been attributed in part to antigenic variation of surface lipoproteins (Razin 1999), as these lipoproteins appear to be the major antigens targeted by the host humoral immune response (Rocha et al. 2005). MG's *vlhA* (variable lipoprotein and hemagglutinin A, formerly *pMGA*) gene family is one example. The gene family encodes variants of VlhA protein, a lipoprotein of the cell membrane that plays a role in adherence, hemagglutination, and evasion of host immune response (Citti et al. 2005, Markham et al. 1992).

The chicken MG strain R_{low} (low passage, high virulence reference strain), has 43 *vlhA* genes that are present at five loci. Comparison of strain R_{low} to the less virulent vaccine F strain of MG shows great variability in the *vhlA* loci of the genome, and sequence data provide evidence for genomic inversion events, gene duplications, and insertion/deletion events (Szczepanek et al. 2010). A single *vlhA* gene is expressed at a time and expression requires a trinucleotide motif ($[GAA]_{12}$) 5' to its promoter (Glew et al. 1998).

The MG VlhA surface protein undergoes phenotypic variation *in vitro* in response to the addition of VlhA-specific monoclonal antibodies to growth media (Markham et al. 1998). While it is thought that VlhA phenotypic variation is a possible mechanism for host immune evasion, VlhA switching also occurs in the absence of detectable antibodies. Glew et al. (2000) studied MG VlhA switching in chickens by examining colonies of MG grown from post-inoculation tracheal wash samples. They found that by two days post-aerosol inoculation, 40% of MG cells ceased expression of the VlhA surface protein expressed at the time of inoculation; however, VlhA specific antibodies were not detected in serum and tracheal samples until day 6 post-inoculation. Thus, it may be possible that the *vlhA* gene family plays a role in other functions beside antibody evasion, such as colonization (Glew et al. 2000).

Recent work by Tulman et al. (2012) compared the genomes of eight MG isolates collected from free-living house finches at various geographic locations between 1994 and 2008. They found that genomes were highly conserved among the isolates, with most differences involving the *vlhA* gene loci. House finch MG genomes contained 33 to 48 *vlhA* genes, with many genes having homologues at similar loci in poultry strains R and F, although some house finch MG *vlhA* genes lacked homologues in the poultry strains. Variation in VlhA expression among house finch isolates has not been documented.

In a previous study (Grodio et al. 2012), we found that eye lesion severity was positively correlated with pathogen load, serum antibody response, and lachrymal antibody response during MG infection in house finches, and we also found that MG isolates vary in their ability to cause disease and induce an antibody response. The goal of this study was to further examine differences in conjunctival MG load and antibody responses during chronic versus acute self-limiting MG disease in house finches using MG isolates collected from Virginia, North Carolina, and California between 1994 and 2008. To determine if MG VlhA changes were occurring in house finches with chronic infection and disease, Western blots were used to study MG protein expression using post-inoculation mycoplasmal cultures from a sub-set of birds.

7.3. Materials and methods

House finches

In February 2010, 77 house finches of mixed sex and age were captured via cage traps in Maricopa Co., Arizona under permits from Arizona State (SP573456), USFWS (MB158404-1) and the USGS Bird Banding Lab (23513), shipped via commercial air to Virginia Tech, and quarantined for three weeks prior to experimental inoculation. Birds were captured in Arizona because there had been no documented cases of MG in this population (e.g., Bonneaud et al. 2012). All birds were housed in individual cages at constant day length (12L:12D) and temperature and provided with drinking water and pelleted diet *ad libitum* (Daily Maintenance Diet, Roudybush Inc., Woodland, CA). All procedures for animal care and use were approved by Virginia Tech's Institutional Animal Care and Use Committees.

Mycoplasma gallisepticum *isolates*

MG field isolates were acquired from the Mycoplasma Diagnostic and Research Laboratory at the NC State University College of Veterinary Medicine. Mycoplasma isolates were obtained from conjunctival swabs of free-ranging house finches showing clinical signs of conjunctivitis, identified as pure cultures of MG by immunofluorescence, and stored at -70°C. MG isolates selected for use as inocula were expanded in culture using Frey's medium with 15% swine serum (modified from Kleven 1998) incubated at 37°C to log phase then frozen at -70°C in 1 ml aliquots. Viable counts were determined by the most probable number method (Meynell and Meynell 1970). See Table 7.1 for isolates used in experimental inoculation. Upon thawing, isolates were diluted in Frey's media to match the CCU (color changing units)/ml of VA1994.

Table 7.1: *Mycoplasma gallisepticum* (MG) isolates used in experimental infection

Isolate	Origin	Year	Passage number	Identifier	Number of birds inoculated
VA1994	Virginia	1994	7[A]	7994-1 7P 5/24/04	10
NC1995	North Carolina	1995	5[A]	13295-2-5P	11
NC1996	North Carolina	1996	3[A]	1596-4-3P 1/13/10	11
NC2006	North Carolina	2006	4[A]	2006.080-5-4P	11
NC2008	North Carolina	2008	3[A]	2008.031-4-3P	11
CA2006[B]	California	2006	5	2006.052-5P	NP[B]

[A]Passage number used in experimental inoculation. An additional passage in culture was required to obtain enough protein for use in Western blot analysis

[B]See Grodio et al. (2012) for experimental infection data. This isolate was included in VlhA Western blot analysis in the present study. NP = experimental inoculation not performed in this study.

Experimental challenge

Individually-housed birds were challenged by applying 50 µl containing approximately 1.1 x10^6 CCU/ml of MG to each palpebral conjunctivae. Ten birds were inoculated with VA1994. NC1995, NC1996, NC2006, and NC2008 treatment groups each included 11 birds (see Table 7.1 for details). Ten birds served as negative controls and received 50 µl of media alone in each conjunctiva.

Eye lesion score

Birds were examined for eye lesions at days 2 and 7, and weekly thereafter up to twelve weeks post-inoculation. Lesions were scored on a 0 to 3 scale (Sydenstricker et al. 2005) as follows: 1 = minor swelling around the eye, 2 = moderate swelling and eversion of the conjunctival tissue, and 3 = the eye is nearly hidden by swelling and crusted exudates.

MG load

A quantitative PCR assay was used to measure MG load in conjunctival swabs before challenge, and on days 2, 7, 14, 21, 28, 42, and 56 post-inoculation. Sterile forceps (Fisher Scientific, Pittsburgh, PA) were used to hold the lower palpebra and a cotton-tipped wooden-handle swab (Fisher Scientific) dipped in tryptose phosphate broth (TPB) was inserted into the lower palpebral sac. Immediately after sample collection, the tip of the swab was placed into 300 µl of sterile TPB. The swab was swirled and wrung out on the inside of the tube to remove liquid from the swab before discarding. Samples were frozen at -20°C prior to DNA extraction. DNA extraction was performed as per Grodio et al. (2012). Quantitative PCR utilizing the *mgc2* gene of MG was performed as per Grodio et al. (2008 and 2012), except that it was performed using a MyiQ Single Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA) with the following parameters: 95°C for 3 min and 40 cycles of: 95°C for 3 sec and 60°C for 30 sec with a ramp rate at 0.5 degrees/sec. Output values of *mgc2* were summed for both eyes within individuals and sampling dates, and $\log_{10}$ transformed prior to statistical analysis.

Two negative control birds tested qPCR positive in one eye on a single day post-inoculation (n = 1 on day 14 and n = 1 on day 21). Because these individuals were not found to be positive at any other point throughout the experiment and the detected pathogen levels were

very low (log 2.24-3.08) and limited to one eye, these results were assumed to reflect sampling or assay error rather than exposure to MG.

Serum antibodies

Blood was collected from the brachial vein into lithium-heparinized microcapillary tubes, pre-inoculation, at weeks 1, 2, and 3 post-inoculation, and every other week thereafter. Tubes were spun in a tabletop centrifuge and plasma was collected and frozen at -20°C. MG-specific antibodies were measured with Idexx FlockChek *Mycoplasma gallisepticum* antibody ELISA kit utilizing a modified protocol described by Grodio et al. (2012).

Tear IgA

Lachrymal fluids were collected and analyzed for weeks 8 and 10 post-inoculation using modified Schirmer tear test strips (Schering-Plough Animal Health, Union, NJ) as described by Grodio et al. (2009). After collection, tear strips from the right and left eye were placed in a single microcentrifuge tube containing 500 μl of PBS/0.05% Tween 20 (PBS-T). Tubes were vortexed vigorously, tear strips were discarded, and samples were stored at -20°C until assayed. MG-specific IgA antibodies in the lachrymal fluids were measured using the Idexx FlockChek *Mycoplasma gallisepticum* antibody ELISA kit and house finch IgA-specific antisera as described previously (Grodio et al. 2009, Grodio et al. 2012).

Isolation of MG post-inoculation

At weeks 7, 9, and 12 post-inoculation, conjunctival swabs were collected for mycoplasmal culture from three birds in each MG isolate group. Birds had eye lesions at the time of swab

collection. Conjunctival swabs were placed in Remel MicroTest M5 (Thermo Fisher Scientific, Lenexa, KS) transport media. Cultures were expanded using Frey's medium with 15% swine serum (modified from Kleven 1998) and viable counts were determined by the most probable number method (Meynell and Meynell 1970).

Immunoblots

Broth cultures of MG isolates used as inocula (Table 7.1) and post-inoculation isolates were centrifuged for 30 min at ~18,000g and 4°C. The cells were washed three times with sterile PBS and after centrifugation, supernatant was removed and pellets were placed on ice. Washed cells were solubilized in a solution containing RIPA buffers (buffer I: 20 mM Tris-HCl, 0.1% SDS, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, protease inhibitor (Roche Diagnostics, Indianapolis, IN), buffer II: 20 mM Tris-HCl, 0.1% SDS, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA) and benzonase nuclease. Protein concentrations were determined using BCA protein assay (Thermo Scientific, Rockford, IL).

Solubilized proteins of the MG isolates (ranging from 1-3 μg/well as detailed in results section) were diluted 1:1 with sample buffer (4% SDS, 20% glycerol, 50 mM Tris, pH 6.8, 0.1% bromophenol blue, 10% 2-mercaptoethanol) and incubated at 100°C for 10 minutes. Protein ladders utilized in this study include SuperSignal Enhanced MW protein ladder and Spectra multi-color broad range protein ladder (Thermo Scientific). Protein samples and ladders were run on SDS-PAGE gels consisting of 5% stacking/7.5% resolving gels. Replicate gels were created and total protein staining was performed with Imperial Protein Stain (Thermo Scientific) using the manufacturer's instructions.

Proteins were transferred to 0.45 μm nitrocellulose membranes (Bio-Rad, Hercules, CA). Membranes were incubated in blocking buffer (Tris-buffered saline with 1% Tween 20 [TBS-T] plus 5% non-fat dry milk [NFDM, Nestle, Vevey, Switzerland]) for 2 h at room temperature. Mouse monoclonal MAb86, specific for MG VlhA (kindly provided by Dr. Philip Markham, University of Melbourne, Markham et al. 1992), was used at a 1:1000 dilution in TBS-T plus NFDM. After washing, the blot was then incubated with anti-mouse IgG-HRP, heavy and light chain (Bethyl Laboratories, Montgomery, TX) at a 1:30,000 dilution in TBS-T plus NFDM. Blots were washed and SuperSignal WestPico Chemiluminescence substrate (Thermo Scientific) was applied as recommended by the manufacturer. The blots were covered with plastic wrap and exposed to film (Amersham Hyperfilm ECL, GE Healthcare, Piscataway, NJ) in a dark room.

Statistical analyses

Birds were categorized as either "chronic" or "recovered" based on the presence or absence, respectively, of eye lesions at week 10 (Table 2). We then used mixed models in SAS 9.0 (SAS Institute, Cary, NC) in order to test whether disease status ("chronic" or "recovered" based on presence of eye lesions at week 10) predicted differences in pathogen load, serum antibodies, or tear IgA. Our models included the following fixed effects: disease status, days post-inoculation, isolate treatment, and all two-way interactions between these variables. We included bird ID as a random effect in all models in order to account for the fact that we included multiple values from the same individuals over the course of the trials.

Virulence (severity of disease) varies among MG isolates. Hawley et al. (in preparation) has characterized virulence differences among the isolates from the experiments described in this study. In order to control for differences in virulence among the isolates used, isolate identity and

isolate in interaction with disease status were included in the model. We were therefore able to examine the role of disease status in predicting pathogen load, serum antibodies, and tear IgA when variation among isolates (in the tendency to cause chronic disease) was accounted for.

We excluded two individuals from our analyses whose pre-inoculation ELISA values were above the cut-off for positivity calculated in Hawley et al. (2011); however, all results were qualitatively identical when these individuals remained in the analysis.

7.4. Results

Birds with chronic disease

Birds with chronic disease had significantly higher pathogen loads than recovered individuals (Fig. 7.1B; disease status: $F_{1, 40} = 90.8$; $p < 0.0001$; disease status*days post-inoculation: $F_{7, 308} = 36.2$; $p < 0.0001$). Additionally, birds with chronic disease had significantly higher levels of MG-specific serum antibodies (Fig. 7.1C; disease status: $F_{1, 40} = 6.1$; $p = 0.017$; disease status*days post-inoculation: $F_{6, 264} = 8.8$; $p < 0.0001$) and tear IgA (Fig. 7.1D; disease status: $F_{1, 40} = 22.3$; $p < 0.001$; disease status*days post-inoculation: $F_{1,44} = 12.0$; $p = 0.001$). Consistent with previous work (Grodio et al. 2012, Hawley et al. 2010), days post-inoculation was a significant main effect in the models of pathogen load ($F_{7, 308} = 339.3$, $p < 0.0001$), serum antibodies ($F_{6, 264} = 44.3$, $p < 0.0001$), and tear IgA ($F_{1, 44} = 4.5$, $p = 0.0391$).

Because MG virulence varies across isolates, we included isolate in our models as a main effect and in pairwise interaction with disease status and days post-inoculation. Isolate identity was a significant predictor of pathogen load both alone ($F_{4, 40} = 5.0$, $p = 0.002$) and in interaction with days post-inoculation ($F_{28, 308} = 1.5$, $p = 0.04$) and disease status ($F_{4, 40} = 3.7$, $p = 0.013$). Interestingly, isolate identity did not significantly predict serum antibodies (all $F < 1.42$, $p >$

0.13) or tear IgA (all $F < 1.43$, $p > 0.24$) either as a main effect or in interaction with disease status or days post-inoculation.

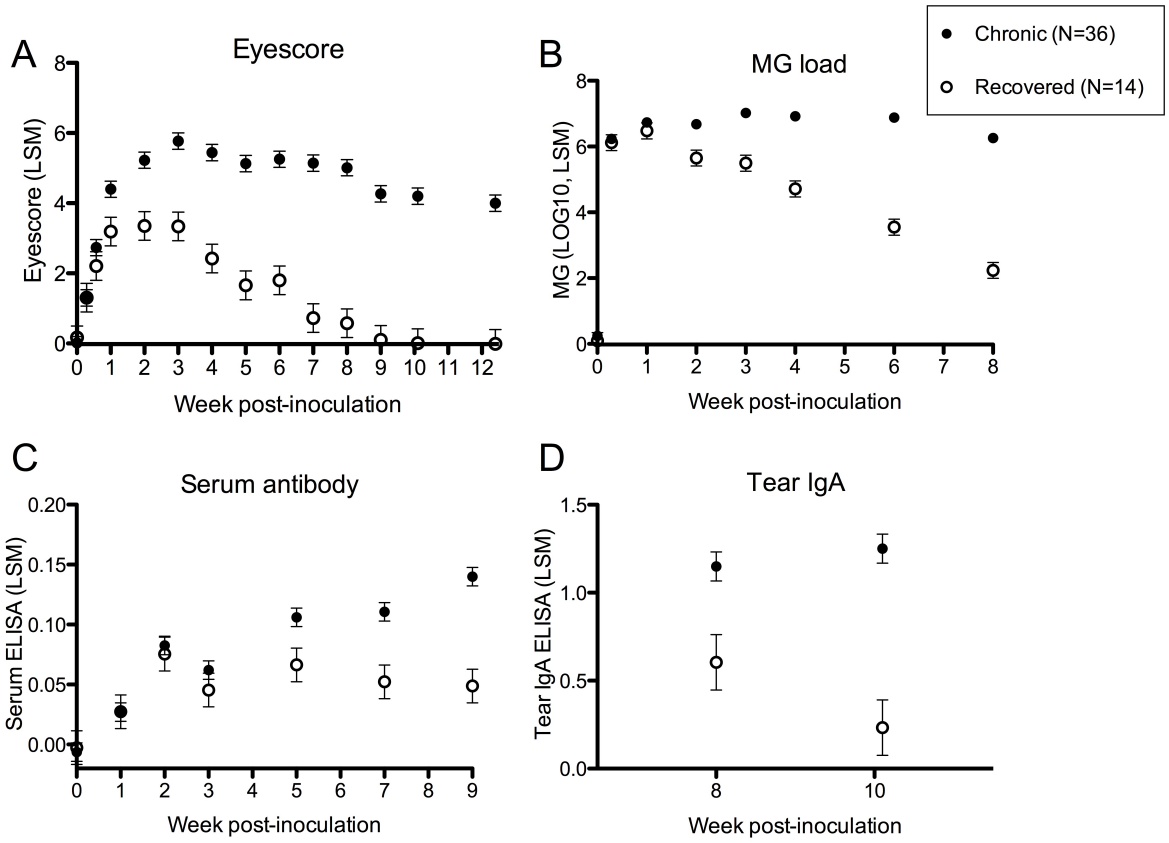

Fig. 7.1. Eyescore, *Mycoplasma gallisepticum* (MG) load, MG-specific serum antibody and tear IgA are shown after experimental inoculation with MG. Least square means (LSM) were calculated to control for variation among MG isolates used in this study. Standard error bars are shown.

mAb86 recognition of house finch MG VlhA protein

Total proteins of inocula and post-inoculation isolates were extracted from broth cultures, quantified, and separated on SDS-PAGE gels. The proteins were transferred to nitrocellulose and incubated with mAb86, anti-mouse IgG-HRP, and substrate. A summary of MAb86 positive

inocula and post-inoculation isolates is listed in Table 7.2. Total protein staining using a Coomassie-based stain is shown in Fig. 7.2A, and confirms the presence of protein in all lanes. As shown in Fig. 7.2B, mAb86 only recognized NC2006 VlhA protein of approximately 50 kDa based on Supersignal ladder. Due to the high intensity of the signal in preliminary blots, a smaller sample of NC2006 protein (1 μg) was utilized in the blot shown compared to the other isolates (3 μg each). When overexposed to film, a very low intensity band of ~40 kDa was observed in inoculum and a few post-inoculation isolates of VA1994 and NC1996; these results had poor reproducibility and are not shown.

Table 7.2: *Mycoplasma gallisepticum* expression of MAb86+ VlhA protein

Isolate	Chronic[A]	MAb86 + inoculum	MAb86+ Week 7	MAb86+ Week 9	MAb86+ Week 12
CA2006	-[B]	-	NP[B]	NP[B]	NP[B]
VA1994	4/10	-	0/3	0/3	0/3
NC1995	6/11	-	1/3	2/3	2/3
NC1996	9/11	-	0/3	0/3	0/3
NC2006	9/10	+	3/3	3/3	1/2
NC2008	9/10	-	0/3	0/3	0/3

[A] Number of birds with lesions still present at week 10 post-inoculation, out of the total number of birds in experimental group.

[B] See Grodio et al. (2012) for experimental inoculation data. NP = post-inoculation mycoplasmal cultures were not performed.

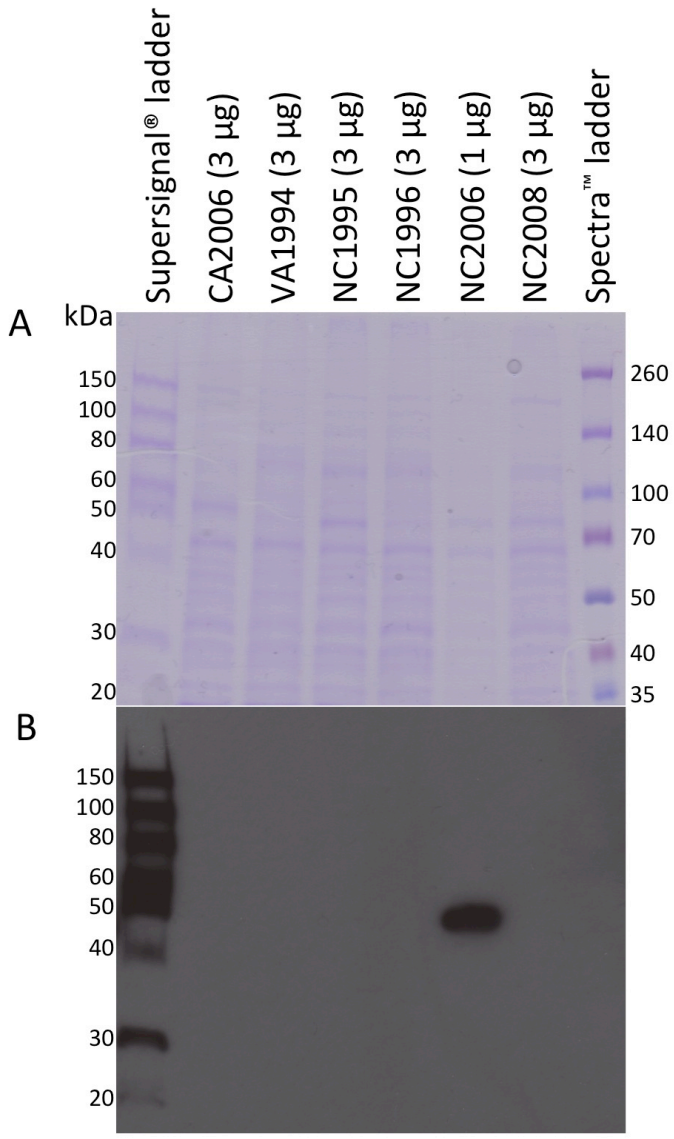

Fig. 7.2. VlhA and total protein staining of six MG isolates. Proteins of six *Mycoplasma gallisepticum* isolates were separated by size using SDS-PAGE (1 µg NC2006, 3 µg of others). Total protein was stained and shown in A. In B, monoclonal antibody MAb86 was used to identify MG VlhA protein. Because NC2006 protein had high intensity staining with MAb86, only 1 µg protein was used for this isolate.

VlhA expression changes during infection with NC2006

Fig. 7.3A shows total proteins using Coomassie-based stain and confirms the presence of protein in all lanes (2 μg protein samples were used). Fig. 7.3B shows high intensity staining of a VlhA variant in the inoculum and lower-intensity staining in the post-inoculation isolates, demonstrating that expression of the VlhA variant at the time of inoculation decreased post-inoculation in all birds tested. Week 12 for bird 163 is not shown because the MG culture was negative at this time-point.

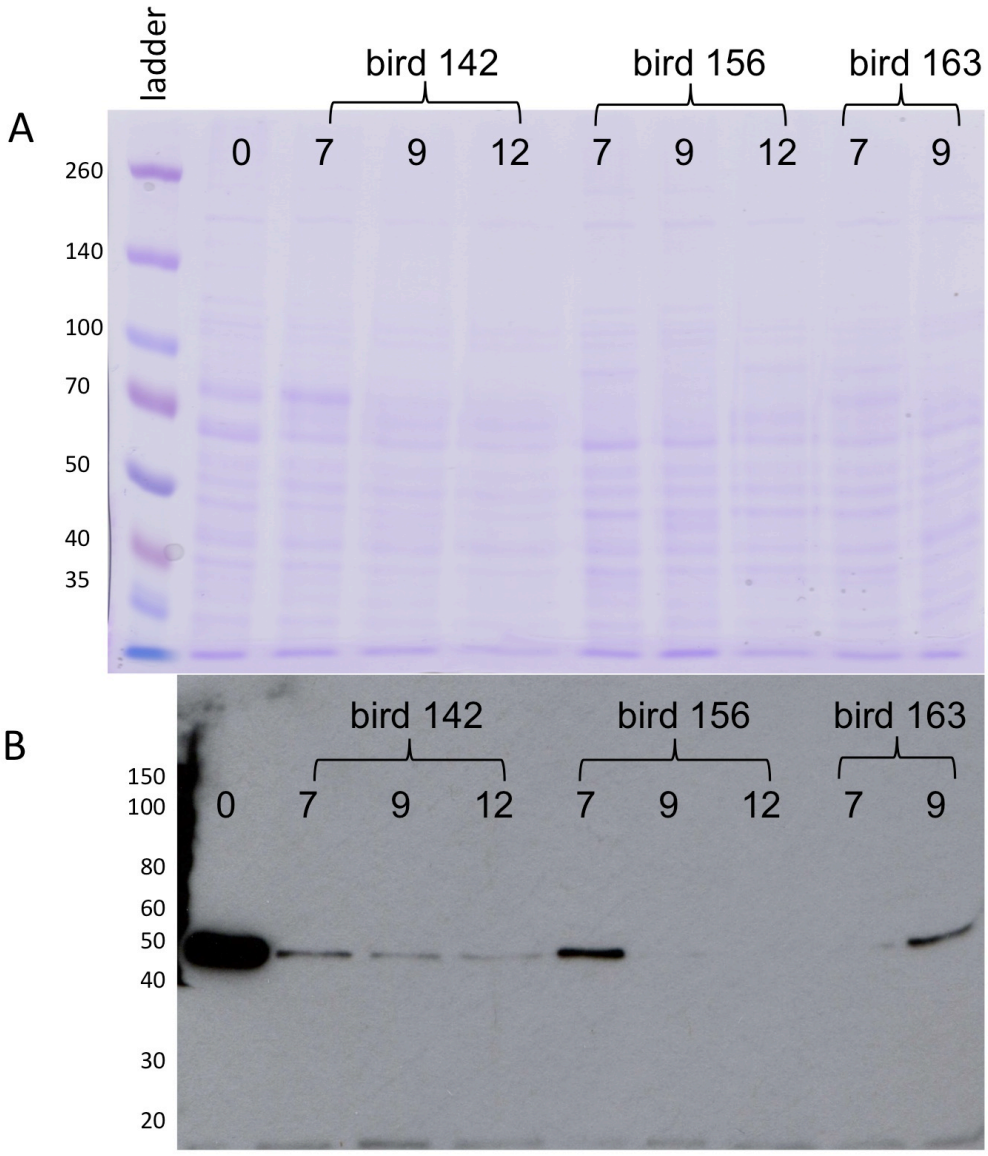

Fig. 7.3. NC2006 VlhA expression changes post-inoculation. Protein samples (2 µg) of *Mycoplasma gallisepticum* (MG) isolate NC2006 were separated by size using SDS-PAGE. 0 = NC2006 pre-inoculation. 7, 9, 12 = NC2006 post-inoculation MG cultures from conjunctival swabs of three birds at 7, 9, and 12 weeks post-inoculation. MG culture for bird 163 at week 12 was negative. Spectra^{TM} ladder was used in A. In B, overexposed Supersignal ® ladder was present to the left of sample "0."

VlhA expression changes during infection with NC1995

Fig. 7.4A shows total proteins using a Coomassie-based stain and confirms the presence of protein in all lanes (2 μg protein samples were used). Fig. 7.4B shows mAb86 staining of a VlhA variant. The original inoculum has no staining for VlhA using MAb86. In two out of three birds tested (birds 146 and 153), expression of the VlhA variant recognized by mAb86 is acquired. In bird 153, this change occurred between week 7 and 9 post-inoculation. In bird 146, expression of the VlhA variant recognized by mAb86 occurred prior to week 7 post-inoculation.

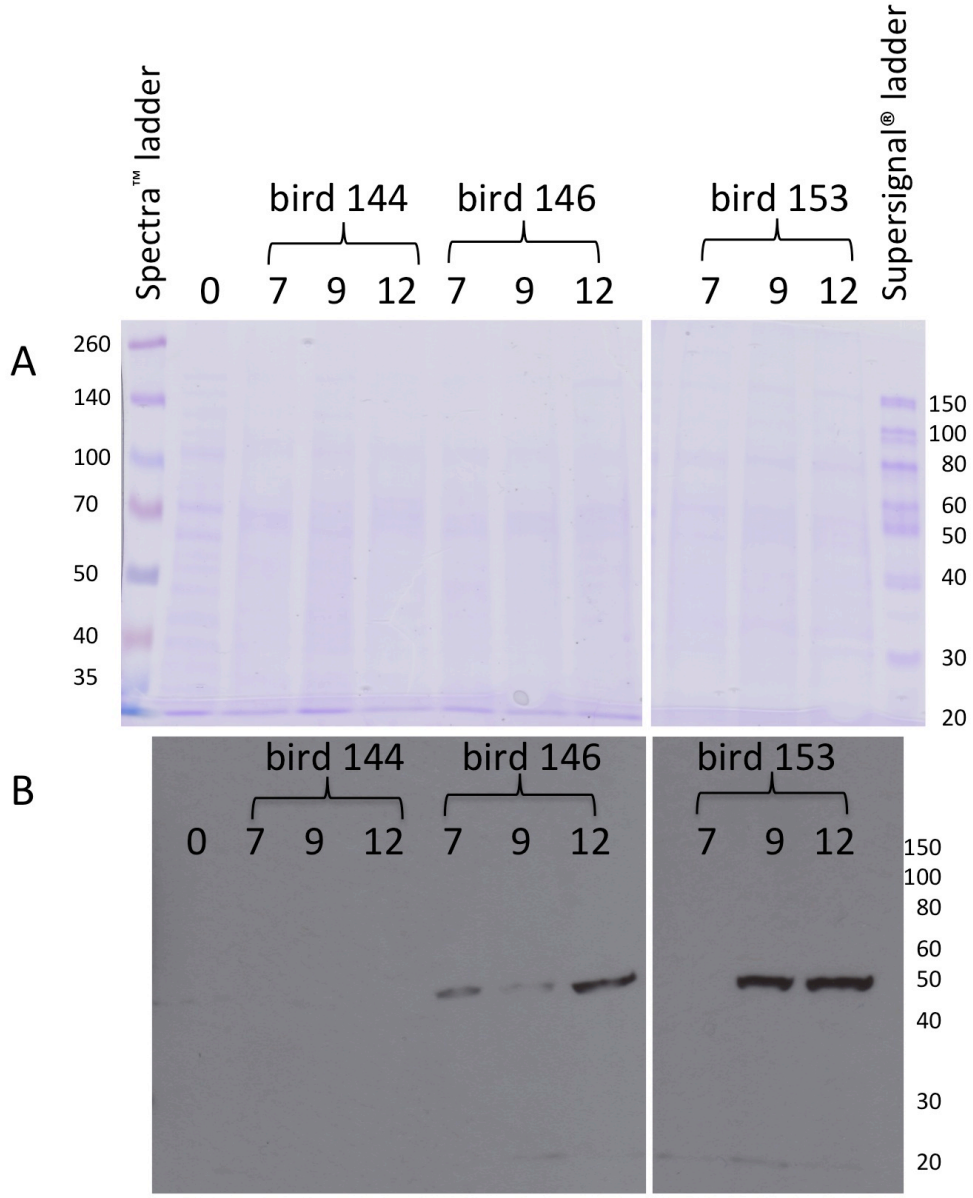

Fig. 7.4. NC1995 VlhA expression changes post-inoculation. Protein samples (2 μg) of *Mycoplasma gallisepticum* (MG) isolate NC1995 were separated by size using SDS-PAGE. 0 = NC1995 pre-inoculation. 7, 9, 12 = NC1995 post-inoculation MG cultures from conjunctival swabs of three birds at 7, 9, and 12 weeks post-inoculation.

7.5. Discussion

Birds with chronic conjunctivitis had high conjunctival loads of MG in spite of high levels of MG-specific serum and tear antibodies. These findings are consistent with those of Grodio et al. (2012), showing that a small percentage (15%) of house finches infected with MG isolate CA2006 developed severe chronic disease characterized by high levels of pathogen and MG-specific antibodies. A larger percentage of birds in this study (40-90%, see Table 2) developed chronic disease compared to birds experimentally inoculated with CA2006. This may be explained by the choice of isolates used in this study, as Hawley et al. (in preparation) found that more recent eastern isolates (e.g. NC2006, NC2008) have increased virulence compared to earlier eastern isolates and western isolates.

In order to control for the possibility that isolate differences in virulence underlie the detected differences between chronic and recovered birds, we included isolate in our statistical models. As shown previously (Hawley et al. 2010, Grodio et al. 2012), isolate identity was a significant predictor of pathogen load both alone and in interaction with days post-inoculation. We also detected a significant interaction between isolate identity and disease status indicating that the influence of disease status on pathogen load may vary across isolates. Interestingly, isolate identity was not a significant predictor of serum antibodies or tear IgA levels. This suggests that chronicity of disease is a more important predictor of antibody differences in the serum and tears than the virulence of the MG isolate.

Birds with chronic disease and infection may play an important role in the spread of MG to other susceptible individuals, since these birds presumably remain infectious for long time periods. Furthermore, diseased individuals spend longer periods of time on bird feeders (Hawley et al. 2007), thus potentially contributing to the spread of MG via fomites (Dhondt et al. 2007).

Quantitative PCR data show that birds with chronic disease tend to harbour MG at very high levels. While qPCR does not distinguish between viable and non-viable organisms, most individuals with clinical signs had positive MG cultures at weeks 7, 9, and 12 post-inoculation, and thus, had viable MG in the conjunctiva up to 12 weeks post-inoculation.

The mouse monoclonal antibody MAb86 specific for MG VlhA recognized a 50 kDa protein of NC2006 and post-inoculation isolates of NC1995. MAb86 likely recognizes only a subset of VlhA proteins because it was created by immunizing mice with membrane suspensions of a single MG strain, poultry strain S6. After subjecting whole S6 cells to SDS-PAGE, Markham et al. (1992) found that MAb86 reacted with a single band of M_r 67,000. MAb86 also reacted to a protein of similar size in strain R, and a protein of M_r 75,000 in strain F. These results suggest that MAb86 may detect at least two VlhA protein variants that share similar epitopes.

NC2006 was MAb86-positive pre-inoculation, but expression of the VlhA variant decreased by seven weeks post-inoculation. In contrast, NC1995 was not MAb86-positive pre-inoculation. In two out of three birds tested, NC1995 gained expression of the VlhA variant detected by MAb86 during the course of infection. Although based on a small sample size, these results show that the VlhA variant expressed at the time of inoculation decreases in expression post-inoculation and that VlhA expression can change up to seven to nine weeks post-inoculation. To the authors' knowledge, only one other study documents MG VlhA expression changes *in vivo* (Glew et al. 2000), and it focused on VlhA changes early (1-28 days) post-inoculation in the chicken. Our results demonstrate the ability of MG to modify cell-surface proteins late in the course of infection in the house finch; alteration of surface proteins could promote chronic infection via evasion of the host antibody response.

Prior to this work and that of Grodio et al. (2012), it was unclear whether chronic disease in house finches is pathogen-mediated or host (immune)-mediated. Existing evidence suggests that both pathogen and immune-mediated mechanisms contribute to mycoplasmal disease. Many *Mycoplasma* species have the ability to vary cell surface antigens, which are the main targets of the humoral immune response (e.g. *vlp* of *M. hyorhinis*, *vsp* of *M. bovis*, *vlhA* of *M. synoviae* and *M. imitans*) (Citti et al. 2005). Since antibody responses are important for controlling extracellular pathogens, the ability to vary cell surface antigens is likely an adaptation for evading the host immune response and establishing prolonged infection.

Studies utilizing immunocompromised hosts provide evidence for both the protective and immunopathologic nature of the adaptive immune response during *Mycoplasma* infection. Cartner et al. (1998) found that SCID (severe combined immunodeficiency) mice infected with *M. pulmonis* developed less severe lung lesions but had similar pathogen loads in the lungs compared to immunocompetent controls, suggesting that the presence of an adaptive immune response increases disease severity. SCID mice had greater quantity of *M. pulmonis* in the spleen and lesions in other extrapulmonary sites when compared to the controls. When SCID mice were given anti-*M. pulmonis* serum, extrapulmonary dissemination was prevented, illustrating the importance of antibodies in controlling spread of infection to other tissues (Cartner et al. 1998).

The method that we used to define chronic individuals is a potential caveat of our study. We used the presence of eye lesions at week 10 (day 71 post-inoculation) as our measure of chronicity. The time course of eye lesions following experimental infection (Fig. 1A) indicates that our method of categorization resulted in two biologically distinct groups of birds, with individuals categorized as “chronic” still harboring equivalently high average lesion scores at week 12 (day 87 post-inoculation) when compared to week 10, the time interval at which we

categorized chronicity of disease. Therefore, our categorization is likely to represent biologically meaningful differences in response to infection. However, future work should consider the most informative and robust way to classify chronic disease in this system.

Our results provide evidence that chronic MG disease in house finches has both immunopathologic and pathogen-mediated components; birds with chronic disease have high pathogen loads in the presence of local and systemic antibody responses that are ineffective at clearing infection, but result in an on-going inflammatory response. Our results also indicate that MG undergoes antigenic changes during infection in the house finch and provides a possible explanation for why the house finch antibody response fails to clear MG infection in cases of chronic disease. Further work should test the hypothesis that evasion of the host immune response drives antigenic changes in MG during *in vivo* infection.

Acknowledgements

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CHAPTER 8

Summary of conclusions

8.1. Quantification of MG genome load in conjunctival samples using real-time PCR

A TaqMan-based real-time, quantitative polymerase chain reaction (qPCR) assay utilizing the *mgc2* gene was developed to detect MG in conjunctival swabs of experimentally infected house finches. The test had a detection limit of less than 14 copies per reaction when tested with a plasmid standard and less than 10 copies per reaction when tested with MG genomic DNA. House finch gene *rag-1* was used as an internal control. The *rag-1* qPCR results showed that host cell quantity varied greatly between conjunctival samples. After inoculation, MG levels in the house finch conjunctiva increased over the 7-day period post infection. The bird with the most pronounced clinical conjunctivitis harbored the highest level of MG and the bird that did not develop conjunctivitis had very low numbers of MG. This small-scale experiment provided the first evidence that development of conjunctivitis may correlate with MG load in house finches.

Since its development, the *mgc2* assay has been used at Cornell and other institutions to study the house finch-MG system (Adelman et al. 2013, Balenger 2011, Bonneaud et al. 2011, Bonneaud et al. 2012, Dhondt et al. 2012, Dhondt et al. 2013, Hawley et al. 2010, Hawley et al. 2011, Hawley et al. in preparation, Hurtado 2012a and 2012b, States 2012).

8.2. Development of house finch IgA-specific anti-sera

House finch IgA-specific anti-sera were developed to measure local (lachrymal) antibody response in subsequent experiments. A 469 base-pair region of the house finch IgA heavy chain was PCR-amplified from spleen cDNA and sequenced. The heavy chain fragment was produced using a bacterial expression system and purified. Rabbit anti-sera were generated against the recombinant protein. The anti-sera reacted with a single house finch serum protein (approximately 50–55 kDa) in Western blots.

The anti-sera were used to identify plasma cells in the Harderian gland and conjunctiva of house finches with conjunctivitis associated with MG infection. The anti-sera also detected IgA+ plasma cells in the Harderian glands of house sparrows and American goldfinches (see also Cushing et al. 2011). The anti-sera were utilized in an ELISA to detect MG-specific IgA antibodies in lachrymal samples of infected finches, and demonstrated that only small amounts (approximately 0.75 mL) of lachrymal fluid are adequate to detect changes in IgA level as infection progressed. While providing a limited amount of sample, collection of tears using modified Schirmer tear strips was simple, quick to perform, and posed no harm to the delicate conjunctival tissues.

8.3. Measuring MG-specific serum antibodies

Prior to this work, serum plate agglutination tests (SPA) were used to screen for the presence of MG-specific antibodies in house finch serum (e.g., Kollias et al. 2004, Sydenstricker et al. 2006). SPA is a common test used to screen poultry flocks for MG infection (Kleven 1998). The test is based on direct visualization of antigen-antibody complexes and does not rely on host-specific secondary antibodies; thus, SPA using commercially available antigen has been used to

document MG infection in house finches. While the SPA is considered a sensitive test, it has low specificity (false positive reactions) (Kleven 1998). Additionally, interpretation can be difficult because test results are based on a visual assessment of the agglutination reaction.

A commercial ELISA (IDEXX, Westbrook, Maine) is available to detect MG-specific serum antibodies in chicken and turkey serum samples. When used as directed with house finch serum samples, the test has low sensitivity (Grodio unpublished results). Several modifications were performed to increase the sensitivity in house finch samples, as described in appendix 1. With modifications, the ELISA has been used to measure MG-specific IgA in house finch tears (Grodio et al. 2009, 2012, submitted) and house finch MG-specific serum antibodies (Hawley et al. 2011, Grodio et al. 2012, Grodio et al. submitted). The modified ELISA has also been used to detect MG-specific serum antibodies in domestic canaries (Hawley et al. 2011, Appendix 2). The main advantages of using the ELISA include: (1) quantitative results, (2) fewer difficulties in interpretation of results, since the plates are read by a spectrophotometer, and (3) less serum is required, therefore samples can be run in duplicate to increase accuracy of results.

8.4. Virulence and immunogenicity of three MG isolates in house finches

Temporal and spatial genotypic variation in MG was documented prior to this work, but phenotypic variation in pathogenicity and immunogenicity was not explored. House finches were inoculated with MG isolates Virginia (VA)1994, California (CA)2006, or North Carolina (NC)2006, which were cultured from free-living house finches with conjunctivitis in 1994, 2006, and 2006, respectively. Infection with NC2006 resulted in the most severe eye lesions, highest pathogen loads, and highest levels of pathogen-specific lachrymal and serum antibodies. Infection with CA2006 caused the least severe eye lesions, lowest pathogen load, and lowest

levels of antibodies. Serum and lachrymal antibody responses were positively correlated with lesion scores, suggesting that the presence of antibodies may contribute to the disease process or that antibodies may be ineffective at clearing MG.

In concordance with past experiments (Kollias et al. 2004, Sydenstricker et al. 2006, Dhondt et al. 2007b, Hawley et al. 2010), the study demonstrated that there is considerable individual variation in response to infection; birds infected with the same MG isolate at identical doses can develop very different courses of disease. All isolates caused severe, protracted disease in a small number of individuals. The three birds with persistent lesions infected with CA2006 had high numbers of MG in spite of developing a very robust antibody response.

Sera from birds infected with NC2006 and VA1994 recognized approximately eight to ten proteins at two weeks post-infection. Sera from birds infected with CA2006 reacted with fewer proteins. ‘‘Heterologous’’ immunoblotting was used to examine the potential for cross-immunity and was performed by separating proteins of a given isolate (e.g., VA1994) and incubating the blot with sera from a bird infected with a different isolates (CA2006 or NC2006). The heterologous immunoblots showed that antibodies directed against one isolate could also recognize proteins of the other isolates, showing that they are antigenically similar; thus, there may be partial cross-protection if a house finch encounters two or more strains of MG throughout the course of its lifetime.

8.5. Chronic MG conjunctivitis in house finches: host antibody response and MG VlhA expression

The study described above showed that MG has the ability to cause persistent, severe infection in a subset of individuals. With an emphasis on chronically infected birds, we further characterized

MG infection using additional field isolates (VA1994, NC1995, NC1996, NC2006, NC2008). Quantitative PCR and ELISAs were used to measure MG load post-infection and house finch antibody response, respectively. Birds with chronic infection had high conjunctival loads of MG in spite of high levels of MG-specific serum and tear antibodies. While qPCR does not distinguish between viable and non-viable organisms, most individuals with clinical signs had positive MG cultures at weeks 7, 9, and 12 post-infection, thus, had viable MG in the conjunctiva up to 12 weeks post-infection.

In Chapter 6, I showed that a small percentage (15%) of house finches infected with MG isolate CA2006 developed severe chronic disease characterized by high levels of pathogen and serum and tear antibodies. A larger percentage of birds in this later study developed chronic infection compared to experimental infection with CA2006. This may be explained by the choice of isolates used in this study, as Hawley et al. (in preparation) found that more recent eastern isolates (e.g., NC2006, NC2008) have increased virulence compared to earlier eastern isolates and western isolates.

Using a monoclonal antibody (MAb86) specific for a variant of the MG VlhA surface protein, it was found that VlhA expression differs among MG isolates and that *in vivo* VlhA changes occur in house finches infected with MG. NC2006 was MAb86-positive pre-inoculation, but expression of the VlhA variant decreased by seven weeks post-infection. In contrast, NC1995 was not MAb86-positive pre-inoculation. In two out of three birds tested, NC1995 gained expression of the VlhA variant detected by MAb86 during the course of infection. Although using a small sample size, these results show that the VlhA variant expressed at the time of inoculation decreases in expression post-infection and that VlhA expression can change up to seven to nine weeks post-infection. To date, only one other study documents MG VlhA

expression changes *in vivo* (Glew et al., 2000), and it focused on VlhA changes early (1-28 days) post-infection in the chicken. These results demonstrate the ability of MG to modify cell-surface proteins late in the course of infection in the house finch; alteration of surface proteins could promote chronic infection via evasion of the host antibody response.

Appendix 2 shows house finch antibody response to MG proteins using samples from two birds also utilized in the VlhA Western blot study. The results show that birds develop tear and serum antibodies to approximately fourteen MG proteins, although a larger sample size would be necessary to confirm these conclusions are more generally applicable.

Prior to this work and that of Grodio et al. (2012), it was unclear whether chronic disease in house finches is pathogen-mediated or host (immune)-mediated. These results suggest that chronic MG disease in house finches has both immunopathologic and pathogen-mediated components; birds with chronic disease have high pathogen loads in the presence of local and systemic antibody responses that are ineffective at clearing infection, but result in an on-going inflammatory response. The results also indicate that MG undergoes antigenic changes during infection in the house finch and provides a possible explanation for why the house finch antibody response fails to clear MG infection in cases of chronic disease.

8.6. Future Considerations: study of chronic infection

8.6.1. MG VlhA

Recent work by Tulman et al. (2012) compared the genomes of eight MG isolates collected from free-living house finches at various geographic locations between 1994 and 2008. They found that genomes were highly conserved between the isolates, with most major differences involving the *vlhA* gene loci. House finch MG genomes contain 33 to 48 *vlhA* genes. Many genes have

homologues at similar loci in poultry strains R and F, although some house finch MG *vlhA* genes lack homologues in the poultry strains. This thesis provides evidence that there is also variation in VlhA expression among house finch isolates, and that changes in MG VlhA expression occur during infection in the house finch.

Now that the genomes of several house finch MG isolates have been sequenced (Tulman et al. 2012), VlhA expression could be more thoroughly investigated with the use of microarray analyses. A microarray could be created using all known house finch MG *vlhA* sequences. Sequence identity among the *vlhA* gene variants in poultry isolates ranges from 41-99% (Papazisi et al. 2003), so the high degree of homology must be taken into account when constructing the microarray (e.g., short vs. long oligonucleotide).

Experimental infections, similar to the ones described in this thesis, could then be carried out to study *in vivo* changes in VlhA expression. After inoculating house finches via conjunctival route, conjunctival swabs would be collected at various time points post-infection and processed for microarray analysis.

Questions that could be addressed with a VlhA microarray include:

1) How does VlhA expression change with passage in culture?
    - This question is important because all isolates studied in experimental infections must be passaged in culture prior to use in experiments. In all previous studies, we have made conclusions based on the assumption that the MG isolates do not change significantly with only a few passages in culture.
2) Which VlhA variant is the MG isolate expressing at the time of experimental inoculation?

- Since we would be testing an isolate with a sequenced genome, we could analyze the genome to find which VlhA variant has the required trinucleotide repeat, $(GAA)_{12}$ 5' to the promoter. It would be expected that the VlhA variant with the specific GAA repeat would be the one expressed at inoculation.

3) When VlhA expression changes post-infection, is a specific VlhA variant preferentially expressed?
    - We would need to compare MG VlhA expression changes occurring in multiple birds to answer this question. Example: In 7 out of 10 birds experimentally infected, MG isolate VlhA switches from type A (VlhA expressed in inoculum) to type B. In 2 out of the 10 birds, MG isolate switches from VlhA type A to type C. In 1 out of the 10 birds, there was no VlhA switch documented, and this bird recovered quickly post-infection.
4) How does VlhA expression vary in birds with chronic disease vs. in birds with disease of shorter duration?
5) Within an individual bird, is one predominating MG VlhA expressed, or are multiple VlhA variants expressed at each time-point?
    - For this question, we consider the *population* of MG infecting an individual bird at any given time-point. Are most members of this population expressing the same VlhA variant, or is it a mixed population expressing several VlhA variants?

8.6.2. Other potential mechanisms for establishing chronic infection

Besides variation of cell surface antigens, MG may have other mechanisms that aid in establishment of chronic infection. There is evidence that MG can form biofilms, although the ability to form biofilms does not necessary correlate with virulence of poultry strains (Chen et al. 2012). Biofilm formation would decrease the ability of the humoral immune system to clear infection and thus could contribute to chronicity of disease. Additionally, as discussed in Chapter 2, there is evidence that MG has the ability to invade nonphagocytic cells (Winner et al. 2000, Vogl et al. 2008), which may also provide a potential mechanism for establishing chronic infection via evasion of the host immune system. The ability of house finch strains of MG to form biofilms and establish intracellular infections could be explored in future research.

8.6.3. Chronic disease and the house finch immune system

This thesis focused mainly on the house finch antibody response to MG because the humoral immune system is very important for control of extracellular pathogens. Other facets of the house finch immune system could also contribute to chronicity of disease, such as components of the innate immune response and production of cytokines.

After experimental inoculation of house finches, Adelman et al. (in press) measured the pro-inflammatory cytokine IL-1β and the anti-inflammatory cytokine IL-10 at 24 hours post-inoculation using quantitative PCR for cytokine mRNA expression; it was found that cytokine levels differed significantly between house finch populations (eastern vs. western), but whether cytokine responses differed between birds with "chronic" vs. "acute" disease was not investigated. Additionally, Bonneaud et al. (2011, 2012) utilized a cDNA microarray to study house finch gene expression changes after experimental inoculation with MG. They documented

expression changes in a number of immune-related genes post-infection, although they explored differences at the population level (eastern vs. western house finch populations) as opposed to investigating differences in birds with chronic and acute disease. Changes in expression of immune-related genes could be examined to identify differences in host response that may contribute to chronicity of disease.

APPENDIX 1

Detection of MG-specific serum antibodies in domestic canaries*

*Text and figure adapted from:
Hawley, D.M., Grodio, J., Frasca, S., Kirkpatrick, L., and D.H. Ley. 2011. Experimental infection of domestic canaries (*Serinus canaria domestica*) with *Mycoplasma gallisepticum*: a new model system for a wildlife disease. *Avian Pathology*. 40: 321-327. Reproduced with permission (no license required).

A1.1. Introduction

Clinical signs of MG are predominately restricted to members of the finch family (Fringillidae) (Hartup et al. 2000, 2001; Mikaelian et al. 2001). Experimental infections by Farmer et al. (2005) confirmed that clinical signs occur in the house finch, American goldfinch (*Carduelis tristis*), pine siskin (*Carduelis pinus*), and purple finch (*Carpodacus purpureus*), all members of the Fringillidae family. The tufted titmouse (*Baelophus bilcolor*), a member of the family Paridae, also showed some evidence of clinical disease after experimental infection.

MG infection in the domestic canary (*Serinus canaria domestica*), member of the Fringillidae family, was not evaluated prior to this work. Since the canary is commonly bred in captivity and commercially available, it has the potential to serve as a model species in captive studies of MG. Only results and discussions related to canary antibody response are reported below. See Hawley et al. (2011) for additional details.

A1.2. Methods

The experimental infection was performed at Virginia Tech, in Dr. Dana Hawley's laboratory. Seven domestic canaries were purchased from a private breeder. Three house finches were captured from Montgomery County, Virginia (n=1) and Tompkins County, New York (n=2) under appropriate permits. Seven canaries and two house finches were inoculated with MG isolate NC2006. One house finch served as a negative control.

Conjunctival swabs were collected on days 7, 14, 21, 28, and 35 post-infection for measurement of MG load. Quantitative PCR was performed by the Hawley lab using a protocol developed by Grodio et al. (2008). Blood was collected from the brachial vein, once prior to inoculation and also on days 14, 21, 28, and 35 post-infection. Plasma was separated and frozen

at -20°C until shipment to Cornell for processing.

The IDEXX FlockChek MG ELISA kit (IDEXX, Westbrook, Maine, USA) was used to measure serum antibodies according to the manufacturer's protocol with modifications. A blocking step consisted of adding 300 ml of 1% bovine serum albumin in phosphate-buffered saline (Pierce 10X BSA; Thermo Fisher Scientific, Rockford, Illinois, USA) to room temperature plates and incubating for 40 minutes. All washing steps consisted of washing the plate three times with 350 ml phosphate-buffered saline with 0.05% Tween 20 using ELx50 Auto Strip Washer (Bio-Tek, Winooski, Vermont, USA). Serum samples were diluted 1:50 in sample buffer, and incubated for 1 hour. Pre-diluted kit antibody was also incubated for 1 hour. Absorbance was measured at 630 nm using an ELx800 Universal Microplate Reader (Bio-Tek). Samples were run in duplicate and the average was calculated for further analysis. To control for inter-assay variation, an ELISA value was calculated based on the equation: (sample mean - negative control)/(positive control - negative control).

A1.3. Results

Using mixed general linear model (JMP 8.0, SAS Institute), there was no significant effect of species on antibodies ($F_{1,6.9} = 0.30$; $P = 0.60$) nor the interaction of species and days post-infection on antibodies ($F_{1,31.1} = 0.01$; $P = 0.91$). However, the number of days post-infection was statistically significant for antibody levels ($F_{1,31.1} = 10.8$, $P = 0.0025$), indicating that this measure of infection varied with time post-infection.

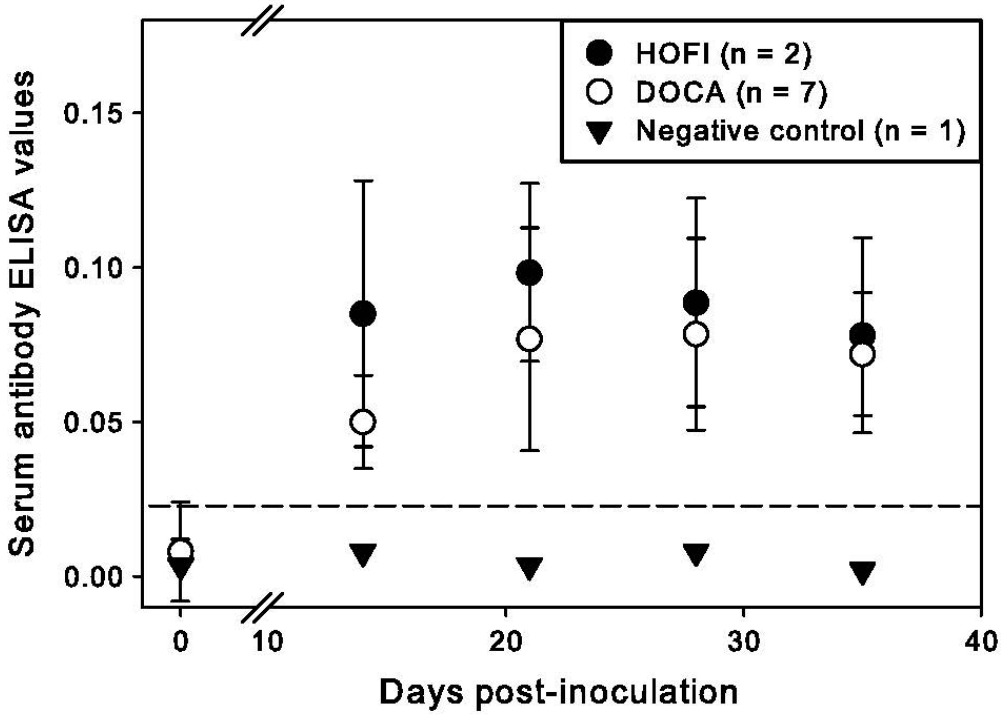

Fig. A1.1. Serum antibody ELISA values for house finches (HOFI) and domestic canaries (DOCA) following experimental inoculation with MG. The dotted line indicates an approximate cut-off value (0.0229). The cut-off value (2.5 standard deviations above the mean) was determined using ELISA values from 108 uninfected house finches that tested negative for MG by qPCR (J. Grodio & D. Hawley, unpublished results).

A1.4. Discussion

Antibody responses in our study were measured using a modified protocol of a commercially available IDEXX kit designed for detecting MG-specific antibodies in chicken sera. This kit has been successfully used in house finches for detecting MG-specific IgA antibodies in the lacrimal fluid (Grodio et al. 2009). To detect house finch serum antibodies, we introduced several modifications to the IDEXX protocol that were aimed at increasing the sensitivity of the test (e.g., using less dilute sera). We have consistently observed elevated optical densities after

experimental infection of house finches with MG (J. Grodio, unpublished); thus, the kit reagent specific for chicken immunoglobulin cross-reacts with house finch immunoglobulin. The results of this study show that the reagent also cross-reacts with canary immunoglobulin. Although the mean optical densities for canaries were lower on average than for house finches, serological responses did not differ statistically between the two songbird species. Larger sample sizes are needed in order to determine whether the sensitivity or specificity of the IDEXX kit differs between these two species.

APPENDIX 2

House finch antibody response to *M. gallisepticum* studied by Western blotting

A2.1. Introduction

In Chapter 7, I show that MG undergoes antigenic variation of the VlhA surface protein during infection of several birds inoculated with MG isolates NC2006 and NC1995. This appendix shows that bird 142, inoculated with NC2006, and bird 153, inoculated with NC1995, develop antibodies to approximately fourteen MG proteins. Using serum and tear samples from bird 142, Western blot shows that antibodies present in the serum and tears are specific for a similar array of MG proteins.

A2.2. Methods

Experimental inoculation

House finches 142 and 153 were inoculated via ocular route by application of inoculum (NC2006 and NC1995, respectively) as detailed in Chapter 7.

MG protein sources

Post-inoculation MG isolates were collected as detailed in Chapter 7. Briefly, conjunctival swabs were collected from birds at 7, 9, and 12 weeks (days 49, 63, and 87) post-inoculation. Swabs were placed in transport media and MG cultures were expanded to obtain enough protein for use in Western blotting. Proteins were extracted and measured as detailed in Chapter 7.

Serum and tear samples

Blood samples were collected pre-inoculation and at 7, 9, and 12 weeks (days 49, 63, and 87) post-infection. Tear samples utilized in the Western blots were collected at weeks 7 and 9 post-infection (see Chapter 7 for details).

Immunoblots

SDS-PAGE was performed as per Grodio et al. (2012, Chapter 6) and Grodio et al. (submitted, Chapter 7) using 2 μg of MG protein per lane. After transfer to nitrocellulose, individual lanes were cut from the blot and blocked using 5% non-fat dry milk. After washing, the blots were then incubated with sera (diluted 1:200) or tears (approximately 0.6 uL tears per blot) of inoculated house finches. After incubation, the blots were washed. The blots that were incubated with house finch serum were then incubated with goat anti-bird IgG-HRP (Bethyl Laboratories, Montgomery, TX), diluted 1:60,000 in blocking buffer for 1 h at room temperature. The blots that were incubated with tears were then incubated with anti-house finch IgA used at 1:1000 (1.5 hrs; Grodio et al. 2009), and followed by incubation with goat anti-rabbit IgG-HRP (Bethyl) diluted 1:30,000. Blots were washed and SuperSignal WestPico Chemiluminescence substrate (Thermo Scientific) was applied. The blots were covered with plastic wrap and exposed to film (Amersham Hyperfilm ECL) in a dark room.

A2.3. Results and discussion

The immunoblots shown in Fig. A2.1 demonstrate that house finches develop antibodies to approximately fourteen MG proteins. As expected, the pre-inoculation sera showed no antibodies to MG proteins. Although the array of MG proteins recognized by house finch antibodies seems rather constant after infection, Figs. 7.3 and 7.4 (described in Chapter 7) illustrate that VlhA expression is changing in these two individuals.

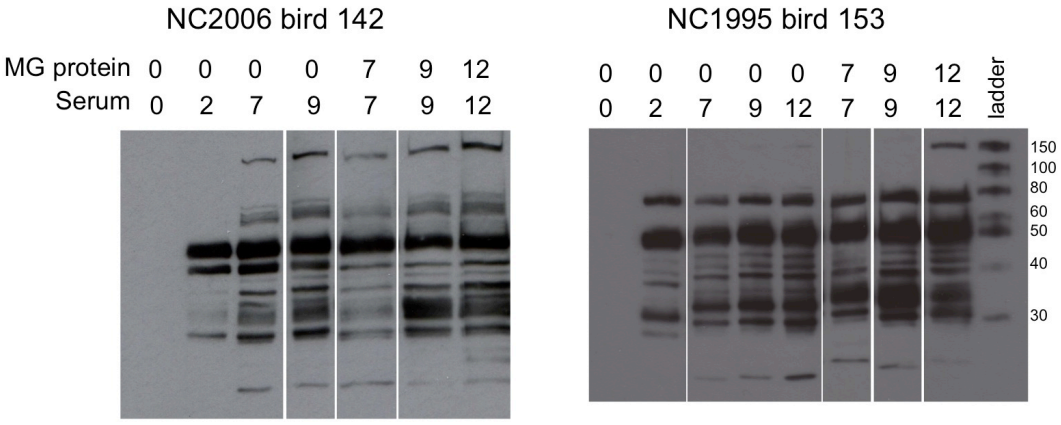

Fig. A2.1. Immunoblots of *Mycoplasma gallisepticum* (MG) proteins incubated with house finch sera. Protein samples (2 μg) of MG isolate NC2006 or NC1995 were separated by size using SDS-PAGE. MG protein 0 = inoculum, 7, 9, 12 = post-inoculation MG cultures from conjunctival swabs of birds at 7, 9, and 12 weeks post-inoculation. House finch serum 0 = pre-inoculation, 2, 7, 9, 12 = weeks post-inoculation.

Using serum and tear samples from bird 142, Fig. A2.2 shows that MG-specific serum IgY and tear IgA antibodies have very similar patterns on Western blot, demonstrating that serum antibodies likely provide a good representation of local antibody response. This result also suggests that collection of lachrymal fluid has the potential to be a less-invasive method to test for MG-specific antibodies compared to blood collection, although a larger sample size would be necessary to confirm these conclusions.

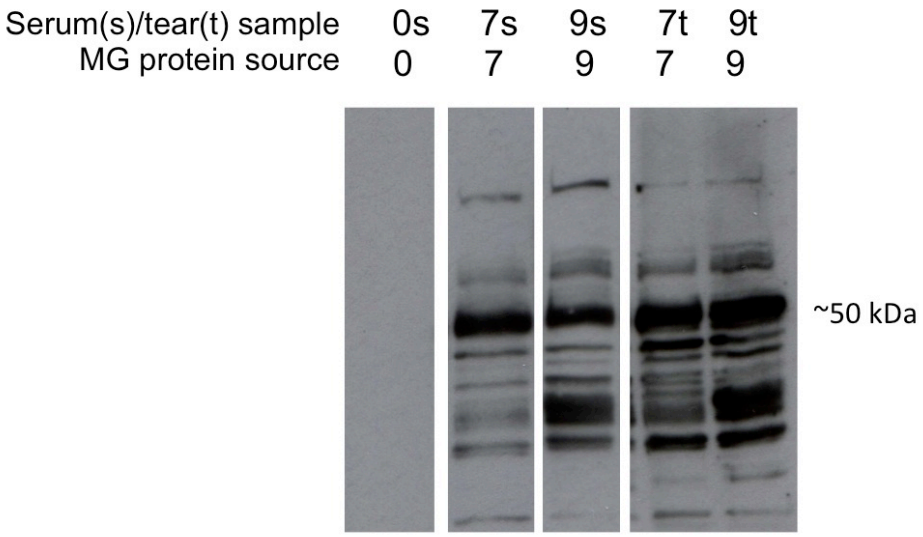

Fig. A2.2. Immunoblots of *Mycoplasma gallisepticum* (MG) proteins incubated with house finch (bird 142) sera and lachrymal fluid. Bird 142 was inoculated with NC2006. MG protein source: 0 = inoculum, 7, 9 = post-inoculation MG cultures from conjunctival swabs at 7 and 9 weeks post-inoculation. Serum/tear samples: 0 = pre-inoculation, 7, 9 = MG-specific serum (IgY) and tear (IgA) antibodies at weeks 7 and 9 post-infection.

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