Mechanisms and Evolution of Disease Resistance in Birds

by

Molly Marie Staley

A dissertation submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

Auburn, Alabama
December 10, 2016

Keywords: house finch, *Haemorhous mexicanus*, *Mycoplasma gallisepticum*, disease resistance, host shift

Copyright 2016 by Molly Marie Staley

Approved by

Geoffrey E. Hill, Chair, Professor of Biological Sciences
Camille Bonneaud, Professor of Evolutionary Biology
Mark R. Liles, Professor of Biological Sciences
Scott R. Santos, Professor of Biological Sciences

Abstract

Disease outbreaks in wildlife, agricultural animals, and humans have increased in both frequency and magnitude in recent decades, refocusing research efforts on understanding the factors that influence disease emergence. However, at least in wildlife, such research is constrained by a lack of knowledge regarding host immune responses to novel pathogens and how those responses may evolve in the face of pathogen driven selection. Consequently, the selective pressures encountered by the pathogen in the novel host, how such pressure may dictate pathogen evolution, and how adaptations to a particular host influence a pathogen's host range are also poorly understood. Here I focus on one of the best-documented wildlife epizootics in history: the emergence of *Mycoplasma gallisepticum* (*Mg*) in wild North American house finches (*Haemorhous mexicanus*) following a host shift from poultry in the mid-1990s. To begin, I review the immune responses wild birds have been shown to mount against novel pathogens and how these responses relate to disease outcome. Subsequently, I examine the house finch-*Mg* system, emphasizing the evolution of house finch immune responses due to *Mg*-driven selection. Second, I expand upon the existing data set regarding the occurrence of *Mg* in house finches near the leading edge of pathogen spread in Arizona, USA. Then, using an experimental infection study with both poultry and early epizootic house finch *Mg* isolates, I show exposure to the novel house finch host was not the key limiting factor in the *Mg* host shift. Lastly, through an experimental infection study where I inoculated chickens with either a poultry or an early epizootic house finch *Mg* isolate, I show adaptation to house finches compromised the ability of

Mg to re-infect poultry. Taken together, the chapters of this dissertation highlight that wild birds are capable of mounting immune responses to novel pathogens such as *Mg* and, in the face of strong-pathogen-driven selection, can undergo rapid evolution of immune responses. Furthermore, *Mg* host shifting was not limited by exposure to the novel host. Rather, genetic adaptation to the novel host environment was likely required at the cost of being able to re-infect poultry.

Acknowledgements

The completion of this dissertation would not have been possible without the invaluable advice and support provided by my colleagues as well as my family and friends. First and foremost, I would like to thank my advisors, Dr. Geoffrey Hill and Dr. Camille Bonneaud, for aiding me in the development and completion of these projects. I also owe a great deal of gratitude to my early research mentors, Dr. Carol Vleck and Dr. Bradley Blitvich, and my Cellular and Molecular Biosciences Program rotation mentors, Dr. Mark Liles and Dr. Kavita Kakirde, for helping me develop the skills set that made this dissertation possible. Furthermore, I would like to thank my collaborators on these projects, Dr. Wendy Hood, Dr. Jon Armbruster, Dr. Carol Vleck, and Dr. Kevin McGraw, for facilitating sample collection, analyses, and the preparation of these manuscripts. Furthermore, I am grateful for the advice and feedback provided over the course of this dissertation by my committee members, Dr. Scott Santos and Dr. Mark Liles, and for Dr. Sarah Zohdy's willingness to serve as my outside reader and provide feedback for improving this work. Lastly, I am indebted to the many Auburn University students and members of the community in both Alabama and Arizona who assisted with obtaining samples for these projects and without whom completing these projects would not have been feasible.

Table of Contents

Abstract ii

Acknowledgments iv

List of Tables viii

List of Figures ix

List of Abbreviations xi

Chapter 1: Immune responses of wild birds to emerging infectious diseases 1

Abstract 1

Introduction 2

Immune responses of wild birds to EIDs 5

Evolution of avian immunity to EIDs: a case study of the outbreak of *Mycoplasma gallisepticum* in house finches 10

Evolution of resistance 10

Insights from studies in poultry 12

Immune processes in the house finch host 14

Evolution of tolerance 17

Conclusion 19

References 21

Box 1 33

Figures 36

Chapter 2: Occurrence of *Mycoplasma gallisepticum* in house finches (*Haemorhous mexicanus*) from Arizona, USA 43

Abstract 43

Introduction 44

Materials and methods 45

House finch sampling 45

Mg presence 46

Results 47

Discussion 48

Acknowledgements 52

References 52

Tables 57

Figures 58

Chapter 3: Testing the role of host exposure in the host shift of an emerging bacterial pathogen 61

Abstract 61

Introduction 62

Materials and methods 65

House finch capture, housing, and experimental infection 65

Quantification of clinical symptom severity 66

MG presence and load 67

Statistical analyses 68

Results 69

Discussion 70

Acknowledgements 73

References 74

Figures 81

Chapter 4: Reduced ability of an emerging pathogen to infect the original donor host following a shift into a novel host 84

Abstract 84

Introduction 85

Materials and methods 89

Chickens, housing, and experimental infection 89

Mg presence 90

Statistical Analyses 91

Results 91

Discussion 93

Acknowledgements 97

References 98

Figures 105

List of Tables

CHAPTER 2

Table 1. Results from 2011 and 2014 house finch sampling in southern and central Arizona .. 57

List of Figures

CHAPTER 1

Figure 1. Experimental WNV infections in American crows (*Corvus brachyrnchos*) and fish crows (*Corvus ossifragus*) 36

Figure 2. Severity of H5N1 HPAI encephalitis in nine naturally infected wild bird species 37

Figure 3. Differential gene expression of HPAI-infected jungle crows 38

Figure 4. House finch with natural *Mycoplasma gallisepticum* and comparison of pathogen load between birds from *M. gallisepticum* exposed and unexposed populations 39

Figure 5. Mass loss and pathogen load in house finches from *M. gallisepticum* exposed and unexposed populations 40

Figure 6. Pathology of house finches from *M. gallisepticum* exposed and unexposed populations 41

Figure 7. Expression of inflammatory cytokines in house finches infected with *M. gallisepticum* and comparison between birds from *M. gallisepticum* exposed and unexposed populations 42

CHAPTER 2

Figure 1. Reported spread of *Mycoplasma gallisepticum* through North America 58

Figure 2. Map of Arizona sampling locations 59

Figure 3. Comparison of house finch displaying conjunctivitis due to *Mycoplasma gallisepticum* infection versus a house finch with suspected avian poxvirus 60

CHAPTER 3

Figure 1. Probability of infection following inoculation with either a poultry strain of *Mg* (Rlow) or house finch epizootic-outbreak isolate (HF1995) 81

Figure 2. Development of clinical symptom in house finches inoculated with either a poultry strain of *Mg* (Rlow) or a house finch epizootic-outbreak isolate (HF1995).................. 82

Figure 3 Bacterial load in the respiratory tract of house finches inoculated with a poultry strain of *Mg* (Rlow) or house finch epizootic-outbreak isolate (HF1995)................................... 83

CHAPTER 4

Figure 1. Probability of infection following inoculation with either a poultry strain of *Mg* (Rlow) or house finch epizootic-outbreak isolate (HF1994) 105

Figure 2. Probability of developing clinical symptoms following inoculation with either a poultry strain of *Mg* (Rlow) or house finch epizootic-outbreak isolate (HF1994).................... 106

Figure 3. Mass gain (in g) over the course of the experiment for chicken inoculated with either chicken Mg (Rlow), house finch Mg (HF1994) or sham-inoculated controls............. 107

List of Abbreviations

EID	Emerging infectious disease
HPAI	Highly pathogenic avian influenza
WNV	West Nile Virus
IFN	Interferon
IL	Interleukin
IgM	Immunoglobulin M
TLR	Toll-like receptor
MHC	Major histocompatibility complex
KEGG	Kyoto Encyclopedia of Genes and Genomes
RLR	Rig-like receptor
NLR	Nod-like receptor
Rlow	Virulent, poultry strain of *Mycoplasma gallisepticum*
GT5	Attenuated, poultry strain of *Mycoplasma gallisepticum*
MIP-1β	Macrophage inflammatory protein β 1
Cd74	MHC class II-associated invariant chain I1
Lgals2	lectin galactoside-binding solu- ble-2
Pd-11	programmed death ligand 1
tcrb	TCR beta chain
IgJ	immunoglobulin J

Ncf4 neutrophil cyto- solic factor-4

Igsf4A immunoglobulin superfamily member 4A

Ptms parathymosin

hCG40889 complement factor-H

Mg *Mycoplasma gallisepticum*

PCR Polymerase chain reaction

Rlow Virulent poultry strain of *Mycoplasma gallisepticum*

HF1995 Epizootic outbreak strain of house finch *Mycoplasma gallisepticum* collected in Georgia, USA, during 1995.

HF1994 Epizootic outbreak strain of house *Mycoplasma gallisepticum* collected in Virginia, USA, during 1994.

CHAPTER 1

Immune responses of wild birds to emerging infectious diseases

Abstract

Over the past several decades, outbreaks of emerging infectious diseases (EIDs) in wild birds have attracted world- wide media attention, either because of their extreme virulence or because of alarming spillovers into agricultural animals or humans. The pathogens involved have been found to infect a variety of bird hosts ranging from relatively few species (e.g. *Trichomonas gallinae*) to hundreds of species (e.g. West Nile Virus). Here we review and contrast the immune responses that wild birds are able to mount against these novel pathogens. We discuss the extent to which these responses are associated with reduced clinical symptoms, pathogen load and mortality, or conversely, how they can be linked to worsened pathology and reduced survival. We then investigate how immune responses to EIDs can evolve over time in response to pathogen-driven selection using the illustrative case study of the epizootic outbreak of *Mycoplasma gallisepticum* in wild North American house finches (*Haemorhous mexicanus*). We highlight the need for future work to take advantage of the substantial inter- and intraspecific variation in disease progression and outcome following infections with EID to elucidate the extent to which immune responses confer increased resistance through pathogen clearance or may instead heighten pathogenesis.

Introduction

The drastic impact that infectious diseases can have on their hosts is illustrated in humans by records of mortality rates resulting from outbreaks like the Spanish flu pandemic of 1918–1920 (Reid *et al.* 2000), as well as more recently by evidence of the role of pathogens in shaping our genome (Fumagalli *et al.* 2011; Karlsson *et al.* 2014). Emerging and re-emerging infectious diseases (EIDs), which include novel diseases that have spread to a new host species or population and historical diseases that have rapidly increased in incidence (Morse 1995), are particularly strong selection events (Kerr 2012). They can therefore pose significant threats to wild populations through loss of genetic diversity, population declines and even localized extinctions of already endangered species (Daszak *et al.* 2000; Lips *et al.* 2006). Given that risks of disease (re)emergence are thought to be aggravated by anthropogenic factors, ranging from our intensive farming practices to the increased movement of organisms across the globe (Patz *et al.* 2004), it is now urgent to improve our understanding of how hosts respond to novel diseases and how immune processes evolve subsequently.

Over the past century, wild birds have been subject to devastating, yet well-documented, wildlife epizootics (Box 1) (Fischer *et al.* 1997; Hayes *et al.* 2005; Lawson *et al.* 2012; Marra *et al.* 2004; Olsen *et al.* 2006; Warner 1968), making them valuable models for studying host immune responses to EIDs, as well as how pathogen- driven selection shapes the evolution of host immunity. For example, between December 2002 and January 2003, Hong Kong saw large die-offs of new and old world species of ducks, geese and swans from highly pathogenic avian influenza (HPAI) (Ellis *et al.* 2004), Great Britain lost over half a million greenfinches (*Carduelis chloris*) and chaffinches (*Fringilla coelebs*) within 2 years of the emergence of *Trichomonas gallinae* (Lawson *et al.* 2012; Robinson *et al.* 2010), and an estimated hundreds of

millions of house finches (*Haemorhous mexicanus*) in the eastern United States died following the *Mycoplasma gallisepticum* epizootic that began in 1994 (Fischer *et al.* 1997; Ley *et al.* 1996; Nolan *et al.* 1998). Similarly, the emergence of West Nile Virus (WNV) in New York (NY) in 1999 was accompanied by more than 17,000 dead bird sightings between May and November of that year, one-third of which were American crows (*Corvus brachyrhynchos*) (Eidson *et al.* 2001a). The causal role of disease in these observed mortality rates was confirmed through testing of carcasses and sick individuals (Bernard *et al.* 2001; Eidson *et al.* 2001a) followed by experimental infection studies (Brault *et al.* 2004; Brown *et al.* 2008; Komar *et al.* 2003; Pasick *et al.* 2007). American crows experimentally infected with the NY-1999 WNV strain exhibited 100% mortality with severe clinical symptoms before death including anorexia, weight loss, encephalitis, and oral and/ or cloacal haemorrhaging (Brault *et al.* 2004; Komar *et al.* 2003). Likewise, experimental infection with the H5N1 strain of HPAI resulted in 100% mortality of black swans (*Cygnus atratus*), mute swans (*Cygnus olor*), trumpeter swans (*Cygnus buccinator*), whooper swans (*Cygnus cygnus*) (Brown *et al.* 2008) and Canada geese (*Branta canadensis*) (Pasick *et al.* 2007). Of these, some black swans died without ever exhibiting clinical symptoms, while the remaining black swans and mute swans died <24 h after the onset of clinical symptoms that progressively worsened from mild listlessness to severe neurological symptoms including tremors and seizures (Brown *et al.* 2008). The severe impact that recent EID outbreaks have had on wild avian hosts therefore raises the question of these hosts' ability to mount immune responses to novel pathogens, as well as the extent to which immune responses may have allowed the host to fight off and/or clear the infection (Janeway 2005).

Despite the high mortality rates observed following these EID outbreaks, there appears to be marked variation in disease development and outcome among and within host species

(Dhondt *et al.* 2014; Komar *et al.* 2003). For example, between 2007 and 2010, wild-caught individuals from 27 of 53 bird species were found to be or to have been infected with *M. gallisepticum* based on PCR and/or testing of serum for antibodies via rapid plate agglutination, but only house finches, American goldfinches (*Spinus tristis*), purple finches (*Haemorhous purpureus*) and black-capped chickadees (*Poecile atricapillus*) exhibited conjunctivitis (Dhondt *et al.* 2014). Experimental infections with NY-1999 WNV of 25 species of birds rep- resenting 17 orders also revealed interspecific differences with mean peak viremias ranging from 10^2.8 to 10^12.1 PFU/mL, as well as highly variable mortality, even among species with the greatest viremias (Komar *et al.* 2003). In fact, in the same study, pathogen load did not necessarily predict disease outcome: although American crows had 100% mortality with a mean peak viremia of 10^10.2 PFU/mL, three other species, common grackles (*Quiscalus quiscula*), house sparrows (*Passer domesticus*) and blue jays (*Cyanocitta cristata*), reached higher mean viremias yet exhibited mortalities of only 33%, 50% and 75%, respectively (Komar *et al.* 2003). Variation in disease progression and mortality is found not only among species but also within species, even in those species that display noticeably high mortality rates (Atkinson *et al.* 2001; Atkinson *et al.* 1995; Brown *et al.* 2006; Yorinks & Atkinson 2000). For example, the emergence of *Plasmodium relictum* in the Hawaiian islands following the accidental introduction of its mosquito vector (*Culex quinquefasciatus*) in the early 20th century was devastating to populations of some native Hawaiian species, particularly Hawaiian honeycreepers such as the Apapane (*Himatione sanguinea*), Hawaii Amakihi (*Hemignathus virens*) and Iiwi (*Vestiaria coccinea*) (Vanriper *et al.* 1986; Warner 1968). Yet experimental exposure of those species to *P. relictum* revealed that some individuals survived and those that did displayed lower levels of infected circulating erythrocytes and lost less mass than conspecifics that died from the infection

(Atkinson *et al.* 2000; Atkinson *et al.* 1995; Yorinks & Atkinson 2000). Despite clear evidence of inter- and intra-specific variation in susceptibility to EIDs, our understanding of the precise immune mechanisms by which this variation is achieved remains incomplete.

Here we review the immune responses that are mounted by wild birds to EIDs using data garnered from field studies of live birds and carcasses, as well as laboratory-conducted experimental infections. First, we examine the types of immune responses wild birds are able to mount against novel pathogens at the cellular and molecular level, as well as evaluate how inter- and intra-specific variation in immunity can be linked to variation in disease severity and outcome. Examining such variation is essential for identifying the immune processes associated with differences in disease development and outcome as well as predicting whether and how these immune responses may evolve over time. Finally, we build on the well-documented epizootic outbreak of *M. gallisepticum* in North American house finches to illustrate how immune responses can evolve in natural avian populations in response to novel diseases.

Immune responses of wild birds to EIDs

Evidence from both field and laboratory studies indicate that some individuals are able to mount immune responses against EIDs and that these responses may confer long-term protection against secondary exposures. For example, experimental infections of American kestrels (*Falco sparverius*) and dunlin (*Calidris alpina*) with H5N1 HPAI revealed that birds seroconverted and produced detectable levels of specific antibodies by 4–5 days post-infection (dpi) (Hall *et al.* 2011; Hall *et al.* 2009). Similarly, experimental infection of laughing gulls (*Leucophaeus atricilla*) with H5N1 HPAI revealed that the two of six individuals that survived infection produced antibodies against HPAI (Brown *et al.* 2006). In addition, these two surviving

individuals had no gross lesions at necropsy and only mild encephalitis and pancreatitis due to lymphocytic and heterophilic infiltration, respectively. In contrast, the individuals that died following infection dis- played more severe pathology, including widespread petechial haemorrhaging, necrotizing pancreatitis, cerebral neuronal necrosis and necrotizing adrenalitis (Brown *et al.* 2006). This suggests that the humoral response mounted by the two surviving laughing gulls may have played a role in allowing them to limit or even clear the infection. Such production of specific antibodies has been found to persist, giving rise to stronger adaptive immune responses upon re-infection. For instance, wild-caught rock pigeons (*Columba livia*) that had been naturally infected with WNV produced antibodies against the virus for at least 15 months after capture (Gibbs *et al.* 2005). Similarly, WNV antibodies have been shown to persist in fish crows (*Corvus ossifragus*) for at least 12 months (Wilcox *et al.* 2007) and in various raptors for at least 4 years (Nemeth *et al.* 2008), while house sparrows experimentally infected with WNV had detectable antibodies for up to 36 months (Nemeth *et al.* 2009). When re-challenged with WNV at 6, 12, 24 or 36 months post-infection, 52 of 71 house sparrows exhibited ≥4-fold increases in antibody titres and only one individual re-challenged at 12 months post-infection became viremic; all individuals given a primary challenge, in contrast, became viremic (Nemeth *et al.* 2009). In the same way, house finches experimentally re-infected with *M. gallisepticum* 219, 314 or 425 days after the primary infection showed reduced conjunctival swelling and duration of clinical symptoms from 7 dpi onwards relative to the response they exhibited upon primary exposure (Sydenstricker *et al.* 2006).

Differences in the intensity and duration of humoral immune responses to EID may also be associated with variation in disease progression and outcome between avian host species. Experimental infections of American crows and fish crows with WNV revealed that fish crows

showed milder and delayed clinical symptoms as well as lower pathogen loads, and exhibited peak viremias of 10^4.7–6.3 PFU/mL at 3–4 dpi that declined to 10^1.7– 2.2 PFU/mL by 6 dpi, whereas American crows had peak viremias of 10^8.22–9.6 at 4–5 dpi that were still high (10^7.3– 7.7) at 6 dpi (Nemeth *et al.* 2011). Individuals from both species seroconverted at 5 dpi, but fish crows displayed a greater antibody production that went from 87–90% WNV serum neutralizing activity at 5 dpi to 93–100% at 6 dpi, while antibody production was lower in American crows with only 41–69% WNV neutralizing activity at 5 dpi and 69– 79% at 6 dpi (Nemeth *et al.* 2011). Taken together, these results suggest that the stronger antibody response of fish crows to WNV may, at least in part, explain their increased ability to resist WNV infection relative to American crows (Figure 1). Whether this is truly the case is unclear, and explicit links between the intensity of humoral immune responses to EIDs, variation in pathogen load, disease development and outcome remain to be explored further.

The immune responses of wild birds to EIDs do not, however, necessarily give rise to decreased disease severity and a greater ability to clear infection, but may instead be associated with a worsening of clinical symptoms through immunopathology (for example, see (Brojer *et al.* 2009; Forzan *et al.* 2010)). This may be particularly true when infections trigger the activation of an inflammatory response, which can damage host tissue and mediate pathogenesis (Graham *et al.* 2005; Rouse & Sehrawat 2010). Damage from inflammation was, for example, found in HPAI-infected wood ducks (*Aix sponsa*) and laughing gulls that exhibited air sacculitis due to heterophil, lymphocyte and plasma cell infiltration (Brown *et al.* 2006). Geese and swans also displayed mild-to- moderate heterophilic and lymphoplasmacytic inflammation in locations where HPAI antigen was detected (Brown *et al.* 2008). HPAI-infected tufted ducks (*Aythya fuligula*) exhibited encephalitis symptoms that upon necropsy were attributed to gliosis,

neuronophagia and inflammatory lesions associated with macrophage and lymphocyte infiltration (Brojer *et al.* 2009). Furthermore, heterophilic infiltration was observed throughout the respiratory system of these individuals, yet there was no inflammation associated with the virus in the intestines (Brojer *et al.* 2009). Patterns of inflammatory responses associated with sites of EID antigen localization have been observed following both WNV and *T. gallinae* infections (Forzan *et al.* 2010; Neimanis *et al.* 2010; Robinson *et al.* 2010; Weingartl *et al.* 2004). For instance, in response to WNV, both blue jays and American crows displayed mixed inflammatory reactions and spleen congestion due to inflammatory cell aggregates and fibrin deposition in areas of inflammation (Weingartl *et al.* 2004). Inflammation in wild finches that succumbed to *T. gallinae* infections in Canada (purple finches) and Great Britain (greenfinches and chaffinches) was found to result from mixed responses of heterophils, macrophages and lymphocytes (Forzan *et al.* 2010; Robinson *et al.* 2010). Such inflammatory responses were also responsible for the mucosal thickening seen in *T. gallinae*-infected greenfinches and chaffinches in Fennoscandia (Neimanis *et al.* 2010). Finally, post-mortem examination of wild birds naturally infected with H5N1 revealed variation in the distribution and severity of the inflammation of the brain, with species exhibiting some of the highest mortality rates from infection (i.e. swans and geese) also displaying the most severe encephalitis, while other species typically showed only mild-to-moderate encephalitis (Figure 2) (Brojer *et al.* 2012; Brown *et al.* 2006; Brown *et al.* 2008; Keawcharoen *et al.* 2008; Kwon *et al.* 2010). All these examples suggest that, in some cases, immune responses (i.e., inflammation) may be detrimental to the host and mediate/accelerate disease progression and outcome. Further support for such a hypothesis comes from the fact that pathogens have been found to benefit from activating inflammatory responses, for example when inflammation disrupts host tissues and facilitates the

infiltration and spread of the pathogen (Hornef *et al.* 2002). Such damages incurred as a result of immune responsive- ness are expected to have important consequences for the evolution of immunity to EIDs, with individuals that remain nonresponsive or activate other components of the immune system being selectively advantaged.

While our understanding of the immune responses to EIDs in wild birds mainly consists of measures of anti- body production or inflammation, investigations into the transcriptomic changes following controlled experimental infection reveal a more complex picture. Huang and colleagues recently compared the global gene expression profiles of lungs from mallards (*Anas platyrhynchos*) infected with H5N1 HPAI to control individuals at 1, 2 and 3 dpi (Huang *et al.* 2013). The number of differentially expressed genes ranged from 2257 to 3066, depending on the day of measurement post-infection and analysis of these genes revealed complex expression patterns of genes known to play roles in immunity. For example, H5N1-infected ducks showed a marked increase (between 2- and 1414-fold) in the expression of five interferon (IFN), 10 chemokine, and 10 interleukin (IL) or IL-receptor genes. The expression of genes known to be involved in the mammalian response to avian influenza and thought to be involved in the avian response including DDX58, IFITM3 and IFIT1–IFIT3, increased between 6.9- and 440-fold, peaking at 2 dpi (Huang *et al.* 2013). Additionally, H5N1-infected mallards exhibited increased expression of two RNA helicases, IFN-induced proteins, Toll-like receptors (TLRs) and major histocompatibility complex (MHC) genes. In contrast, other genes, including immunoglobulin M (IgM), three T-cell receptor (TCR) genes, and 4 CD molecule-encoding genes were shown to have decreased expression (Huang *et al.* 2013). Taken together, these data suggest EIDs elicit altered expression of multiple immune pathways in infected avian hosts.

Such a hypothesis of multiple immune pathways being involved in the responses of wild birds to EIDs is further supported by analyses of H5N1 HPAI-infected jungle crow (*Corvus macrorhynchos*) lung transcriptomes at 6 dpi, which revealed significant differential expression of 2297 genes between infected and control individuals. Based on gene ontology analysis, the majority of differentially expressed genes were found to have immune-associated functions, with other affected genes being involved in cellular metabolism, transcriptional and translational regulation, apoptosis and phagocytosis (Vijayakumar *et al.* 2015). Vijayakumar and colleagues expanded on these findings using Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway analysis to refine the specific crow immunological pathways affected by HPAI infection (Figure 3). For instance, crows showed altered expression of multiple innate immune signaling pathways that are involved in viral recognition and influence activation of adaptive responses such as rig-1-like receptor (RLR) and Nod-like receptor (NLR) signaling pathways. Furthermore, infected crows demonstrated altered expression of genes involved in inflammation including cytokines and chemokines as well as involved in adaptive immunity including TCR signaling (Vijayakumar *et al.* 2015). While gene expression analyses such as those obtained from HPAI-infected mallards and crows represent important advances in our understanding of the immune responses of wild birds to EIDs, they also highlight the complexity of these responses and the gaps in our understanding of the extent to which these responses allow the host to fight and/or clear infection.

Evolution of avian immunity to EIDs: a case study of the outbreak of *Mycoplasma gallisepticum* in house finches

Evolution of resistance

Few novel EID outbreaks in natural populations are as well documented as the *M. gallisepticum* epizootic in North American house finches (Dhondt *et al.* 2006; Farmer *et al.* 2005; Hartup *et al.* 2001b; Hochachka & Dhondt 2000). *Mycoplasma gallisepticum*, an endemic bacterial pathogen of poultry, was first detected in house finches in Maryland in 1994 (Ley *et al.* 1996). Although this bacterium readily switches hosts between chickens (*Gallus gallus*) and turkeys (*Meleagridis gallapovo*), a single lineage of poultry origin has since been confirmed to be responsible for the house finch outbreak (Delaney *et al.* 2012; Hochachka *et al.* 2013). In house finches, *M. gallisepticum* manifests as an upper respiratory tract and eye (conjunctivitis) infection (Ley *et al.* 1996) that can lead to death, in part, through blindness-induced starvation and predation. Following reports of individuals with swollen eyes at birdfeeders, the Cornell Laboratory of Ornithology set up a Citizen Science program (http://www.birds.cornell.edu/hofi/), through which volunteer birdwatchers could report observations of dis- eased house finches (Dhondt *et al.* 1998). This allowed for thorough documentation of both temporal and spatial changes in disease prevalence over time (Hartup *et al.* 2001a). Within 4 years, *M. gallisepticum* had spread throughout house finch populations in the eastern United States, killing an estimated tens of millions of house finches (Fischer *et al.* 1997; Nolan *et al.* 1998). Prevalence, however, subsequently declined from epizootic to apparent enzootic levels (Hartup *et al.* 2001a; Hochachka & Dhondt 2000), raising important questions regarding the possible evolution of resistance/tolerance in house finches and underlying changes in host immune processes.

Investigations of host immune responses at epizootic onset, as well as how these responses subsequently evolved, are made possible in this system due to the persistence of unexposed house finch populations with which to compare infected populations (Bonneaud *et al.* 2011). In 2007, Bonneaud et al. experimentally infected wild-caught finches from disease-

unexposed, western US (Arizona) populations and from disease-exposed, eastern US (Alabama) populations with *M. gallisepticum* to test whether resistance had spread in eastern house finch populations (Bonneaud *et al.* 2011). After verification that the finches had never been naturally infected with *M. gallisepticum*, finches were either inoculated with a contemporary 2007-Alabama strain or sham-inoculated (controls). Two weeks post-infection, finches from disease-unexposed populations harboured nearly 50% greater bacterial loads than finches from exposed populations (Figure 4). Comparison of splenic transcriptional responses to infection of finches from unexposed vs. exposed populations measured before and after the apparent spread of host resistance confirmed that disease-exposed house finch populations had evolved resistance to *M. gallisepticum* from standing genetic variation in only 12 years of disease exposure (Bonneaud *et al.* 2011).

Insights from studies in poultry

Mycoplasma gallisepticum is an economically important bacterium known to infect a wide range of hosts of agricultural relevance (Stipkovits & Kempf 1996), primarily chickens and turkeys. As a result, studies conducted in poultry have pro- vided important insights into the pathogenesis of *M. gallisepticum*, as well as into the immune processes activated in the poultry host. These, in turn, improve our understanding of the host and pathogen processes taking place in the house finch host. In common with other Mycoplasmas (Chambaud *et al.* 1999; Razin *et al.* 1998), *M. gallisepticum* displays the ability to evade and manipulate the immune system of its hosts, with both potentiating and suppressive effects on various components of immunity (Browning & Citti 2014). Teasing apart the immune processes under host and pathogen control and their role in resolving or benefiting infection is therefore challenging. However, insights into

bacterial-driven processes can be obtained, for example, by comparing the immune responses elicited in poultry by closely related virulent and attenuated strains of *M. gallisepticum* (Mohammed *et al.* 2007).

The establishment of infection (i.e. colonization) by *M. gallisepticum* encompasses both adherence to host tissues and initial multiplication, and occurs at the mucosal surface of the respiratory epithelium. This is made difficult by the presence of mucus and mucociliary clearance (Jordan *et al.* 2007). Thus, to facilitate invasion, *M. gallisepticum* can use specific lipoproteins/lipopeptides that bind to host epithelial cells (Chambaud *et al.* 1999) and can induce a misdirected inflammatory response that will disrupt the epithelial membrane (Ganapathy & Bradbury 2003; Gaunson *et al.* 2006). Lesions in host tissues have been shown to result from the recruitment, activation and proliferation of heterophils and macrophages initially, and of lymphocytes subsequently, to and at the site of infection (Gaunson *et al.* 2000). Inoculations of chickens with virulent (Rlow) and attenuated (GT5) strains revealed that this leucocyte chemotaxis is achieved through the release of chemokines by infected tissues (Mohammed *et al.* 2007), including lymphotactin, CXCL13, CXCL14, RANTES and macrophage inflammatory protein b 1(MIP-1b) (Mohammed *et al.* 2007). MIP-1b secretion by chicken monocytes and macrophage-like cells was also confirmed in vitro (Lam & DaMassa 2000) and shown to act as an attractant for many leucocytes, including heterophils, T lymphocytes and NK cells (Lam & DaMassa 2000; Menten *et al.* 2002). Such findings are consistent with the infiltration of nonspecific CD8+TCR0 cells (most likely NK cells) in the tracheal mucosa of infected chickens, with infiltration peaking 1 week post-infection and thought to play an important role in disease progression through cytotoxicity (Gaunson *et al.* 2000, 2006). Comparison of tracheal expression patterns following infection with Rlow and GT5 also confirmed that chickens up-regulated pro-

inflammatory cytokines (Mohammed *et al.* 2007), such as TNF-a and IL-6, which are responsible for local and systemic inflammation and can also give rise to tissue destruction and local necrosis. While the induction of an inflammatory response may therefore be beneficial to *M. gallisepticum* and facilitate invasion of the host (d'Hauteville *et al.* 2002; Hornef *et al.* 2002), persistence of infection may on the other hand necessitate the suppression of other components of immunity (Gaunson *et al.* 2006). Accordingly, chickens infected with Rlow down-regulated the tracheal expression of the chemokine CCL20 and cytokines IL-8, IL-1b and IL-12p40 as early as 1 day post-inoculation (Mohammed *et al.* 2007). The fact that these cytokines are also involved in key inflammatory processes (Murphy *et al.* 2012) highlights the complexity of pathogen-mediated manipulation of the host immune system. Furthermore, chickens infected with *M. gallisepticum* displayed lower T-cell activity 2 weeks post-infection (68,70) and lower humoral responses against *Haemophilus gallinarum* (Matsuo *et al.* 1978) or against avian pneumovirus (Naylor *et al.* 1992) when co-inoculated with *M. gallisepticum*. The ability of *M. gallisepticum* to limit humoral and T-cell responses may be crucial for disease progression, as both local antibody-mediated responses and natural killer and cytotoxic T-cell responses have been suggested to play a role in controlling infection in chickens (Gaunson *et al.* 2006).

Immune processes in the house finch host

Comparison of transcriptional changes in the spleen of infected house finches from disease-unexposed/susceptible and disease-exposed/resistant populations revealed significant differences as early as 3 days post-infection (Bonneaud *et al.* 2012b), indicating that the evolution of resistance in exposed populations involved changes in innate immune processes. Two weeks post-infection, susceptible finches from unexposed populations down-regulated

immune-associated genes and, relative to infected finches from exposed populations, exhibited significantly lower levels of transcripts of the following genes (Bonneaud *et al.* 2012b): T-cell immunoglobulin and mucin domain containing 4 (tim4), MHC class II-associated invariant chain I1 (cd74), lectin galactoside-binding soluble-2 (lgals2), programmed death ligand 1 (pd-l1), TCR beta chain (tcrb), immunoglobulin J (IgJ), neutrophil cytosolic factor-4 (ncf4), immunoglobulin superfamily member 4A (Igsf4A) and parathymosin (ptms). The only exception was the complement factor-H (hCG40889) gene, whose expression was up-regulated in infected finches from unexposed populations. However, because hCG40889 is known to restrict activation of the complement cascade (de Cordoba & de Jorge 2008), the overall expression patterns detected suggest that particular components of the immune system were being suppressed in finches from unexposed populations. Infected finches from exposed populations, on the other hand, were able to up-regulate the expression of immune-associated genes 2 weeks post-infection (Bonneaud *et al.* 2012b). Three of the genes up-regulated were as follows: TIM4, which is involved in the differentiation of naive CD4+ T cells into Th2 cells and which plays a role in preventing autoimmunity by mediating the clearance of apoptotic (phosphatidylserine-expressing) antigen-specific T cells after infection (Kobayashi *et al.* 2007); CD74, which plays a role during the assembly of MHC class II molecules (Bertolino & Rabourdin-Combe 1996); NCF4, which plays a role in phagocytosis- induced oxidant production in heterophils (Matute *et al.* 2009). Taken together, these finding suggests that finches from disease- exposed populations have evolved the ability to resist pathogen-induced immunosuppression and supports a role of both innate (e.g. phagocytosis by heterophils) and acquired (e.g. T-cell activity) immune processes in mediating resistance to pathogen spread (Bonneaud *et al.* 2012b).

Protective immunity is expected to evolve only when the costs of resisting infection are lower than those incurred by the infection itself (Boots & Bowers 2004; Boots & Haraguchi 1999). Surprisingly, resistance to *M. gallisepticum* was found to have evolved despite the fact that the short-term energetic costs of immunity were greater than those of pathogenesis (Bonneaud *et al.* 2012a). Disentangling the costs attributable to immune functioning from those incurred from the parasite's presence is challenging in in vivo infection studies involving real pathogens (Owen *et al.* 2010). As a result, most of our understanding of the costs of immunity stems from studies using inert pathogens (Bonneaud *et al.* 2003). However, two unusual features of the *M. gallisepticum*-house finch interaction permitted such a study in this system. First, it is possible to compare the response to infection between finches that are either susceptible or resistant depending on their population of origin (i.e. disease-unexposed or disease-exposed populations, respectively) (Adelman *et al.* 2013; Bonneaud *et al.* 2011). Second, only resistant finches from disease-exposed populations are able to mount a protective immune response, as demonstrated both by the greater bacterial load and the over- all down-regulation of immune-associated genes at early as 3 days post-infection in finches from unexposed populations (Bonneaud *et al.* 2011; Bonneaud *et al.* 2012b). It is important to note, however, that genes associated with innate immunity, and in particular with inflammation, were not specifically examined in this study and hence may have been up-regulated in finches from unexposed populations at the onset of infection. This hypothesis is supported by the findings of Hawley and colleagues (Hawley *et al.* 2012) showing increased levels of IL-6 in house finches 2 days post-inoculation with *M. gallisepticum*, as well as a 2°C increase in body temperature 1 day post-infection with a ~1°C increase persisting over the entire 2-week duration of the experimental infection. Regardless, the greater susceptibility of finches from disease-unexposed population

implies that any potential inflammatory response was not protective and therefore likely reflects pathogenesis.

As expected based on the findings above, infected finches from disease-exposed populations lost 10 times more body mass over the course of 2 weeks than uninfected controls from the same populations, revealing a cost of immunity (Figure 5a) (Bonneaud *et al.* 2012a). Furthermore, infected individuals from the disease-exposed population that lost the most mass and displayed immune-associated gene expression patterns in a direction consistent with greatest protective immunity (i.e. resistance) against *M. gallisepticum*, also harboured the lowest pathogen loads in their conjunctivae (Figure 5b). Conversely, infected finches from disease-unexposed populations lost twice as much body mass as their controls, although this difference was marginal. In addition, in this population, infected individuals that lost the most mass harboured the greatest bacterial load in their conjunctivae, indicating a measurable cost of pathogenesis. Interestingly, the mass lost by infected birds differed significantly between populations, with mass loss being greater in infected finches from exposed populations (Figure 5). This indicates that, counter to predictions, the short-term energetic costs of immunity were greater than those of pathogenesis (Bonneaud *et al.* 2012a). These results therefore high- light the fact that resistance can evolve despite this, pro- vided the fitness consequences of infection are sufficiently detrimental to the host.

Evolution of tolerance

The consequences of pathogen-driven selection on host evolution in this system are made all the more interesting by the fact that resistance was not the only host trait to evolve following epizootic outbreak. Adelman and colleagues (Adelman *et al.* 2013) demonstrated that pathogen

tolerance, which is the ability to limit the damage incurred from a given pathogen load (Raberg *et al.* 2009), also spread in eastern house finch populations following disease exposure. To this end, they caught finches from disease-unexposed western US (Arizona) and disease-exposed eastern US (Alabama) populations in 2010 and experimentally infected them with an *M. gallisepticum* isolate collected in Virginia in 1994 (i.e. at epizootic onset). Given that exposed populations were shown to have evolved resistance to *M. gallisepticum* between 2001 and 2007 (Bonneaud *et al.* 2011), infection with an isolate sampled 16 years earlier ensured that any immunomodulatory effects of the bacteria would be minimized. Tolerance was then assessed using peak levels of pathology (i.e. eye lesions and mass loss) and bacterial load, as well as measures of pathology and bacterial load that incorporated infection duration and intensity (i.e. by measuring the area under the curves of pathology and pathogen load over time). Results showed that finches from the unexposed population had significantly greater peak eye lesions and mass loss than finches from the exposed population despite similar peak pathogen load. In addition, eye lesions also peaked a week later in finches from the exposed population relative to the unexposed one (e.g. peak eye score; unexposed: 4.13 ± 0.48 on day 7; exposed = 5.79 ± 0.14 on day 14) (Figure 6) (Adelman *et al.* 2013).

The heightened tolerance of finches from the *M. gallisepticum*-exposed population was associated with a lower inflammatory response to infection relative to finches from the unexposed population (Adelman *et al.* 2013). Specifically, finches from exposed populations displayed significantly lower levels of IL-1b, but marginally higher levels of IL-10, 24 h post-infection (Figure 7) (Adelman *et al.* 2013). IL-1b is a pro-inflammatory cytokine secreted by macrophages and that plays a key role in the acute phase response (Murphy *et al.* 2012). The difference in the expression of IL-1b between finches from exposed and unexposed populations

thus is likely to be responsible for the delayed and lowered febrile responses of the former (increase in body temperature on day 1 post-infection; exposed: 0.71° ± 0.03 °C; unexposed: 1.44° ± 0.18°C) (Adelman *et al.* 2013).

While resistance and tolerance are often thought of as two alternative evolutionary responses to pathogen-driven selection (Raberg *et al.* 2009; Raberg *et al.* 2007), studies on the house finch-*M. gallisepticum* system indicate that this may not necessarily be the case and that both processes can evolve in conjunction to reduce the overall fitness cost of infection (Adelman *et al.* 2013; Bonneaud *et al.* 2011). Interestingly, that tolerance reduced both inflammation and the severity of clinical symptoms (i.e. eye lesions and mass loss) without decreasing pathogen load (Adelman *et al.* 2013) suggests that infection success is not necessarily positively correlated with the level of immunopathology suffered by the host. As a result, the extent to which host lesions can be minimized without impacting pathogen colonization success or persistence will determine the relative contribution of resistance and tolerance to the evolutionary response of house finches *to M. gallisepticum*, with significant ramifications for the evolution of pathogen virulence (Raberg *et al.* 2009).

Conclusion

Wild birds have been shown to mount immune responses to emerging infectious pathogens, but these responses are not always associated with reduced severity, or even absence, of clinical symptoms, nor do they necessarily allow the host to clear and survive the infection. The extent to which these immune responses help to fight novel pathogens, however, seems dependent on the type of response elicited, with humoral responses conferring some level of protection and inflammatory responses being associated with increased disease severity. Whether

immune processes allow the host to fight the infection or, on the opposite, facilitate disease progression will have important consequences for the evolution of immune responses over time in response to pathogen-mediated selection. In cases where inflammation underlies disease pathology, a lack of immune responsiveness with or with- out the involvement of other components of immunity (e.g. humoral immunity) may be favoured by natural selection, thus leading to the evolution of tolerance and/ or resistance. The combined spread of tolerance and resistance to EIDs appears to have occurred in house finches following the outbreak of the conjunctivitis-causing *M. gallisepticum*. Whether this evolutionary change was mediated solely by changes in the finches' inflammatory response, with important consequences for more pathogen-specific components of immunity (e.g. T-cell and humoral immunity), or whether host evolution has occurred through parallel changes in multiple components of immunity (e.g. inflammation and T-cell immunity con- currently) remains to be determined. Finally, further insights into the role of different immune processes can be gained from detailed inter- and intraspecific comparisons linking immune responses to EIDs at a molecular and cellular level with variation in disease progression and outcome. By increasing our understanding of the role of host immune responses in EID outbreaks and persistence, studies conducted on wild bird populations will have the potential to improve our predictions of species particularly at risk of infection by EIDs.

References

Adelman JS, Kirkpatrick L, Grodio JL, Hawley DM (2013) House finch populations differ in early inflammatory signaling and pathogen tolerance at the peak of *Mycoplasma gallisepticum* infection. *American Naturalist* **181**, 674-689.

Atkinson CT, Dusek RJ, Woods KL, Iko WM (2000) Pathogenicity of avian malaria in experimentally-infected Hawaii Amakihi. *J Wildl Dis* **36**, 197-204.

Atkinson CT, Lease JK, Drake BM, Shema NP (2001) Pathogenicity, serological responses, and diagnosis of experimental and natural malarial infections in native Hawaiian thrushes. *Condor* **103**, 209-218.

Atkinson CT, Saili KS, Utzurrum RB, Jarvi SI (2013) Experimental evidence for evolved tolerance to avian malaria in a wild population of low elevation Hawai'i 'Amakihi (Hemignathus virens). *Ecohealth* **10**, 366-375.

Atkinson CT, Woods KL, Dusek RJ, Sileo LS, Iko WM (1995) Wildlife disease and conservation in Hawaii: pathogenicity of avian malaria (Plasmodium relictum) in experimentally infected iiwi (Vestiaria coccinea). *Parasitology* **111 Suppl**, S59-69.

Bernard KA, Maffei JG, Jones SA*, et al.* (2001) West Nile virus infection in birds and mosquitoes, New York State, 2000. *Emerg Infect Dis* **7**, 679-685.

Bertolino P, Rabourdin-Combe C (1996) The MHC class II-associated invariant chain: a molecule with multiple roles in MHC class II biosynthesis and antigen presentation to CD4+ T cells. *Crit Rev Immunol* **16**, 359-379.

Bonneaud C, Balenger S, Russell AF*, et al.* (2011) Rapid evolution of disease resistance is accompanied by functional changes in gene expression in a wild bird. *Proc Natl Acad Sci U S A* **108**, 7866-7871.

Bonneaud C, Balenger SL, Hill GE, Russell AF (2012a) Experimental evidence for distinct costs of pathogenesis and immunity against a natural pathogen in a wild bird. *Mol Ecol* **21**, 4787-4796.

Bonneaud C, Balenger SL, Zhang J, Edwards SV, Hill GE (2012b) Innate immunity and the evolution of resistance to an emerging infectious disease in a wild bird. *Mol Ecol* **21**, 2628-2639.

Bonneaud C, Mazuc J, Gonzalez G*, et al.* (2003) Assessing the cost of mounting an immune response. *American Naturalist* **161**, 367-379.

Boots M, Bowers RG (2004) The evolution of resistance through costly acquired immunity. *Proceedings of the Royal Society of London Series B-Biological Sciences* **271**, 715-723.

Boots M, Haraguchi Y (1999) The evolution of costly resistance in host-parasite systems. *American Naturalist* **153**, 359-370.

Brault AC, Langevin SA, Bowen RA*, et al.* (2004) Differential virulence of West Nile strains for American crows. *Emerg Infect Dis* **10**, 2161-2168.

Brojer C, Agren EO, Uhlhorn H*, et al.* (2012) Characterization of encephalitis in wild birds naturally infected by highly pathogenic avian influenza H5N1. *Avian Dis* **56**, 144-152.

Brojer C, Agren EO, Uhlhorn H*, et al.* (2009) Pathology of natural highly pathogenic avian influenza H5N1 infection in wild tufted ducks (Aythya fuligula). *J Vet Diagn Invest* **21**, 579-587.

Brown JD, Stallknecht DE, Beck JR, Suarez DL, Swayne DE (2006) Susceptibility of North American ducks and gulls to H5N1 highly pathogenic avian influenza viruses. *Emerg Infect Dis* **12**, 1663-1670.

Brown JD, Stallknecht DE, Swayne DE (2008) Experimental infection of swans and geese with highly pathogenic avian influenza virus (H5N1) of Asian lineage. *Emerg Infect Dis* **14**, 136-142.

Browning GF, Citti C (2014) *Mollicutes: molecular biology and pathogenesis* Caister Academic Press, Norfolk, UK.

Chambaud I, Wroblewski H, Blanchard A (1999) Interactions between mycoplasma lipoproteins and the host immune system. *Trends in Microbiology* **7**, 493-499.

Chen H, Smith GJ, Zhang SY*, et al.* (2005) Avian flu: H5N1 virus outbreak in migratory waterfowl. *Nature* **436**, 191-192.

Chi JF, Lawson B, Durrant C*, et al.* (2013) The finch epidemic strain of *Trichomonas gallinae* is predominant in British non-passerines. *Parasitology* **140**, 1234-1245.

d'Hauteville H, Khan S, Maskell DJ*, et al.* (2002) Two msbB genes encoding maximal acylation of lipid A are required for invasive Shigella flexneri to mediate inflammatory rupture and destruction of the intestinal epithelium. *Journal of Immunology* **168**, 5240-5251.

Daszak P, Cunningham AA, Hyatt AD (2000) Wildlife ecology - Emerging infectious diseases of wildlife - Threats to biodiversity and human health. *Science* **287**, 443-449.

de Cordoba SR, de Jorge EG (2008) Translational mini-review series on complement factor H: Genetics and disease associations of human complement factor H. *Clinical and Experimental Immunology* **151**, 1-13.

Delaney NF, Balenger S, Bonneaud C*, et al.* (2012) Ultrafast evolution and loss of CRISPRs following a host shift in a novel wildlife pathogen, *Mycoplasma gallisepticum*. *PLoS Genet* **8**, e1002511.

Dhondt AA, Altizer S, Cooch EG*, et al.* (2005) Dynamics of a novel pathogen in an avian host: Mycoplasmal conjunctivitis in house finches. *Acta Trop* **94**, 77-93.

Dhondt AA, Badyaev AV, Dobson AP*, et al.* (2006) Dynamics of mycoplasmal conjunctivitis in the native and introduced range of the host. *Ecohealth* **3**, 95-102.

Dhondt AA, DeCoste JC, Ley DH, Hochachka WM (2014) Diverse wild bird host range of *Mycoplasma gallisepticum* in eastern North America. *PLoS One* **9**, e103553.

Dhondt AA, Tessaglia DL, Slothower RL (1998) Epidemic mycoplasmal conjunctivitis in house finches from Eastern North America. *J Wildl Dis* **34**, 265-280.

Eidson M, Komar N, Sorhage F*, et al.* (2001a) Crow deaths as a sentinel surveillance system for West Nile virus in the northeastern United States, 1999. *Emerg Infect Dis* **7**, 615-620.

Eidson M, Miller J, Kramer L*, et al.* (2001b) Dead crow densities and human cases of West Nile virus, New York State, 2000. *Emerg Infect Dis* **7**, 662-664.

Ellis TM, Bousfield RB, Bissett LA*, et al.* (2004) Investigation of outbreaks of highly pathogenic H5N1 avian influenza in waterfowl and wild birds in Hong Kong in late 2002. *Avian Pathol* **33**, 492-505.

Farmer KL, Hill GE, Roberts SR (2005) Susceptibility of wild songbirds to the house finch strain of Mycoplasma gallisepticum. *J Wildl Dis* **41**, 317-325.

Fischer JR, Stallknecht DE, Luttrell MP, Dhondt AA, Converse KA (1997) Mycoplasmal conjunctivitis in wild songbirds: the spread of a new contagious disease in a mobile host population. *Emerg Infect Dis* **3**, 69-72.

Forzan MJ, Vanderstichel R, Melekhovets YF, McBurney S (2010) Trichomoniasis in finches from the Canadian Maritime provinces--An emerging disease. *Can Vet J* **51**, 391-396.

Foster JT, Woodworth BL, Eggert LE*, et al.* (2007) Genetic structure and evolved malaria resistance in Hawaiian honeycreepers. *Mol Ecol* **16**, 4738-4746.

Fumagalli M, Sironi M, Pozzoli U*, et al.* (2011) Signatures of environmental genetic adaptation pinpoint pathogens as the main selective pressure through human evolution. *PLoS Genet* **7**, e1002355. doi:1002310.1001371/journal.pgen.1002355.

Ganapathy K, Bradbury JM (2003) Effects of cyclosporin A on the immune responses and pathogenesis of a virulent strain of Mycoplasma gallisepticum in chickens. *Avian Pathology* **32**, 495-502.

Gaunson JE, Philip CJ, Whithear KG, Browning GF (2000) Lymphocytic infiltration in the chicken trachea in response to *Mycoplasma gallisepticum* infection. *Microbiology-Uk* **146**, 1223-1229.

Gaunson JE, Philip CJ, Whithear KG, Browning GF (2006) The cellular immune response in the tracheal mucosa to Mycoplasma gallisepticum in vaccinated and unvaccinated chickens in the acute and chronic stages of disease. *Vaccine* **24**, 2627-2633.

Gibbs SE, Hoffman DM, Stark LM*, et al.* (2005) Persistence of antibodies to West Nile virus in naturally infected rock pigeons (Columba livia). *Clin Diagn Lab Immunol* **12**, 665-667.

Graham AL, Allen JE, Read AF (2005) Evolutionary causes and consequences of immunopathology. *Annual Review of Ecology Evolution and Systematics* **36**, 373-397.

Hall JS, Franson JC, Gill RE*, et al.* (2011) Experimental challenge and pathology of highly pathogenic avian influenza virus H5N1 in dunlin (Calidris alpina), an intercontinental migrant shorebird species. *Influenza Other Respir Viruses* **5**, 365-372.

Hall JS, Ip HS, Franson JC*, et al.* (2009) Experimental infection of a North American raptor, American kestrel (*Falco sparverius*), with highly pathogenic avian influenza virus (H5N1). *PLoS One* **4**, e7555.

Hartup BK, Bickal JM, Dhondt AA, Ley DH, Kollias GV (2001a) Dynamics of conjunctivitis and *Mycoplasma gallisepticum* infections in house finches. *Auk* **118**, 327-333.

Hartup BK, Dhondt AA, Sydenstricker KV, Hochachka WM, Kollias GV (2001b) Host range and dynamics of mycoplasmal conjunctivitis among birds in North America. *J Wildl Dis* **37**, 72-81.

Hawley DM, DuRant SE, Wilson AF, Adelman JS, Hopkins WA (2012) Additive metabolic costs of thermoregulation and pathogen infection. *Functional Ecology* **26**, 701-710.

Hayes EB, Komar N, Nasci RS*, et al.* (2005) Epidemiology and transmission dynamics of West Nile virus disease. *Emerg Infect Dis* **11**, 1167-1173.

Hochachka WM, Dhondt AA (2000) Density-dependent decline of host abundance resulting from a new infectious disease. *Proc Natl Acad Sci U S A* **97**, 5303-5306.

Hochachka WM, Dhondt AA, Dobson A*, et al.* (2013) Multiple host transfers, but only one successful lineage in a continent-spanning emergent pathogen. *Proc Biol Sci* **280**, 20131068.

Hornef MW, Wick MJ, Rhen M, Normark S (2002) Bacterial strategies for overcoming host innate and adaptive immune responses. *Nature Immunology* **3**, 1033-1040.

Huang Y, Li Y, Burt DW*, et al.* (2013) The duck genome and transcriptome provide insight into an avian influenza virus reservoir species. *Nat Genet* **45**, 776-783.

Janeway C (2005) *Immunobiology : the immune system in health and disease*, 6th edn. Garland Science, New York, NY.

Jordan JL, Chang HY, Balish MF*, et al.* (2007) Protein P200 is dispensable for Mycoplasma pneumoniae hemadsorption but not gliding motility or colonization of differentiated bronchial epithelium. *Infection and Immunity* **75**, 518-522.

Karlsson EK, Kwiatkowski DP, Sabeti PC (2014) Natural selection and infectious disease in human populations. *Nature Reviews Genetics* **15**, 379-393.

Keawcharoen J, van Riel D, van Amerongen G*, et al.* (2008) Wild ducks as long-distance vectors of highly pathogenic avian influenza virus (H5N1). *Emerg Infect Dis* **14**, 600-607.

Kerr PJ (2012) Myxomatosis in Australia and Europe: A model for emerging infectious diseases. *Antiviral Res* **93**, 387-415.

Khan SU, Berman L, Haider N*, et al.* (2014) Investigating a crow die-off in January-February 2011 during the introduction of a new clade of highly pathogenic avian influenza virus H5N1 into Bangladesh. *Arch Virol* **159**, 509-518.

Kobayashi N, Karisola P, Pena-Cruz V*, et al.* (2007) TIM-1 and TIM-4 glycoproteins bind phosphatidylserine and mediate uptake of apoptotic cells. *Immunity* **27**, 927-940.

Komar N, Langevin S, Hinten S*, et al.* (2003) Experimental infection of North American birds with the New York 1999 strain of West Nile virus. *Emerg Infect Dis* **9**, 311-322.

Kramer LD, Bernard KA (2001a) West Nile virus in the western hemisphere. *Curr Opin Infect Dis* **14**, 519-525.

Kramer LD, Bernard KA (2001b) West Nile virus infection in birds and mammals. *Ann N Y Acad Sci* **951**, 84-93.

Kwon YK, Thomas C, Swayne DE (2010) Variability in pathobiology of South Korean H5N1 high-pathogenicity avian influenza virus infection for 5 species of migratory waterfowl. *Vet Pathol* **47**, 495-506.

Lam KM, DaMassa AJ (2000) Mycoplasma gallisepticum-induced release of macrophage inflammatory protein-1 beta from chicken monocytes-macrophages. *Journal of Comparative Pathology* **122**, 35-42.

Lanciotti RS, Roehrig JT, Deubel V*, et al.* (1999) Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. *Science* **286**, 2333-2337.

Lawson B, Robinson RA, Colvile KM*, et al.* (2012) The emergence and spread of finch trichomonosis in the British Isles. *Philos Trans R Soc Lond B Biol Sci* **367**, 2852-2863.

Ley DH, Berkhoff JE, McLaren JM (1996) *Mycoplasma gallisepticum* isolated from house finches (*Carpodacus mexicanus*) with conjunctivitis. *Avian Dis* **40**, 480-483.

Lips KR, Brem F, Brenes R*, et al.* (2006) Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community *Proc Natl Acad Sci U S A* **103**, 3165-3170.

Liu J, Xiao H, Lei F*, et al.* (2005) Highly pathogenic H5N1 influenza virus infection in migratory birds. *Science* **309**, 1206.

Marra PP, Griffing S, Caffrey C*, et al.* (2004) West Nile virus and wildlife. *Bioscience* **54**, 393-402.

Matsuo K, Kuniyasu C, Yamada S, Susumi S, Yamamoto S (1978) Suppression of immuno-responses to Hemophilus gallinarum with nonviable *Mycoplasma gallisepticum* in chickens. *Avian Dis* **22**, 552-561.

Matute JD, Arias AA, Wright NA*, et al.* (2009) A new genetic subgroup of chronic granulomatous disease with autosomal recessive mutations in p40 phox and selective defects in neutrophil NADPH oxidase activity. *Blood* **114**, 3309-3315.

Menten P, Wuyts A, Van Damme J (2002) Macrophage inflammatory protein-1. *Cytokine & Growth Factor Reviews* **13**, 455-481.

Mohammed J, Frasca S, Cecchini K*, et al.* (2007) Chemokine and cytokine gene expression profiles in chickens inoculated with *Mycoplasma gallisepticum* strains R-low or GT5. *Vaccine* **25**, 8611-8621.

Morse SS (1995) Factors in the emergence of infectious diseases *Emerg Infect Dis* **1**, 7-15.

Murphy KP, Travers P, Walport M, Janeway C (2012) *Janeway's immunobiology*, 8th edn. Garland Science, New York.

Naylor CJ, Alankari AR, Alafaleq AI, Bradbury JM, Jones RC (1992) Exacerbation of *Mycoplasma gallisepticum* infection in turkeys by Rhinotracheitis virus. *Avian Pathology* **21**, 295-305.

Neimanis AS, Handeland K, Isomursu M*, et al.* (2010) First report of epizootic trichomoniasis in wild finches (family Fringillidae) in southern Fennoscandia. *Avian Dis* **54**, 136-141.

Nemeth NM, Kratz GE, Bates R*, et al.* (2008) Naturally induced humoral immunity to West Nile virus infection in raptors. *Ecohealth* **5**, 298-304.

Nemeth NM, Oesterle PT, Bowen RA (2009) Humoral immunity to West Nile virus is long-lasting and protective in the house sparrow (Passer domesticus). *Am J Trop Med Hyg* **80**, 864-869.

Nemeth NM, Thomsen BV, Spraker TR*, et al.* (2011) Clinical and pathologic responses of American crows (Corvus brachyrhynchos) and fish crows (C ossifragus) to experimental West Nile virus infection. *Vet Pathol* **48**, 1061-1074.

Nolan PM, Hill GE, Stoehr AM (1998) Sex, size, and plumage redness predict house finch survival in an epidemic. *Proceedings of the Royal Society B-Biological Sciences* **265**, 961-965.

Olsen B, Munster VJ, Wallensten A*, et al.* (2006) Global patterns of influenza a virus in wild birds. *Science* **312**, 384-388.

Owen JP, Nelson AC, Clayton DH (2010) Ecological immunology of bird-ectoparasite systems. *Trends in Parasitology* **26**, 530-539.

Pasick J, Berhane Y, Embury-Hyatt C*, et al.* (2007) Susceptibility of Canada geese (*Branta canadensis*) to highly pathogenic avian influenza virus (H5N1). *Emerg Infect Dis* **13**, 1821-1827.

Patz JA, Daszak P, Tabor GM*, et al.* (2004) Unhealthy landscapes: Policy recommendations on land use change and infectious disease emergence. *Environ Health Perspect* **112**, 1092-1098.

Raberg L, Graham AL, Read AF (2009) Decomposing health: tolerance and resistance to parasites in animals. *Philosophical Transactions of the Royal Society B-Biological Sciences* **364**, 37-49.

Raberg L, Sim D, Read AF (2007) Disentangling genetic variation for resistance and tolerance to infectious diseases in animals. *Science* **318**, 812-814.

Razin S, Yogev D, Naot Y (1998) Molecular biology and pathogenicity of mycoplasmas. *Microbiology and Molecular Biology Reviews* **62**, 1094-1156.

Reid AH, Fanning TG, Janczewski TA, Taubenberger JK (2000) Characterization of the 1918 "Spanish" influenza virus neuraminidase gene. *Proc Natl Acad Sci U S A* **97**, 6785-6790.

Robinson RA, Lawson B, Toms MP*, et al.* (2010) Emerging infectious disease leads to rapid population declines of common British birds. *PLoS One* **5**, e12215.

Rouse BT, Sehrawat S (2010) Immunity and immunopathology to viruses: what decides the outcome? *Nat Rev Immunol* **10**, 514-526.

Sakoda Y, Ito H, Uchida Y*, et al.* (2012) Reintroduction of H5N1 highly pathogenic avian influenza virus by migratory water birds, causing poultry outbreaks in the 2010-2011 winter season in Japan. *J Gen Virol* **93**, 541-550.

Smietanka K, Fusaro A, Domanska-Blicharz K*, et al.* (2010) Full-length genome sequencing of the Polish HPAI H5N1 viruses suggests separate introductions in 2006 and 2007. *Avian Dis* **54**, 335-339.

Stipkovits L, Kempf I (1996) Mycoplasmoses in poultry. *Revue Scientifique Et Technique De L Office International Des Epizooties* **15**, 1495-1525.

Sturm-Ramirez KM, Ellis T, Bousfield B*, et al.* (2004) Reemerging H5N1 influenza viruses in Hong Kong in 2002 are highly pathogenic to ducks. *J Virol* **78**, 4892-4901.

Sydenstricker KV, Dhondt AA, Hawley DM*, et al.* (2006) Characterization of experimental Mycoplasma gallisepticum infection in captive house finch flocks. *Avian Dis* **50**, 39-44.

Vanriper C, Vanriper SG, Goff ML, Laird M (1986) The epizootiology and ecological significance of malaria in Hawaiian land birds. *Ecological Monographs* **56**, 327-344.

Vijayakumar P, Mishra A, Ranaware PB*, et al.* (2015) Analysis of the crow lung transcriptome in response to infection with Highly Pathogenic H5N1 Avian Influenza Virus. *Gene*.

Warner RE (1968) Role of introduced diseases in extinction of endemic Hawaiian avifauna. *Condor* **70**, 101-&.

Weber S, Harder T, Starick E*, et al.* (2007) Molecular analysis of highly pathogenic avian influenza virus of subtype H5N1 isolated from wild birds and mammals in northern Germany. *J Gen Virol* **88**, 554-558.

Weingartl HM, Neufeld JL, Copps J, Marszal P (2004) Experimental West Nile virus infection in blue jays (Cyanocitta cristata) and crows (Corvus brachyrhynchos). *Vet Pathol* **41**, 362-370.

Wilcox BR, Yabsley MJ, Ellis AE, Stallknecht DE, Gibbs SE (2007) West Nile virus antibody prevalence in American crows (Corvus brachyrhynchos) and fish crows (Corvus ossifragus) in Georgia, USA. *Avian Dis* **51**, 125-128.

Woodworth BL, Atkinson CT, Lapointe DA*, et al.* (2005) Host population persistence in the face of introduced vector-borne diseases: Hawaii amakihi and avian malaria. *Proc Natl Acad Sci U S A* **102**, 1531-1536.

Yorinks N, Atkinson CT (2000) Effects of malaria on activity budgets of experimentally infected juvenile Apapane (Himatione sanguinea). *Auk* **117**, 731-738.

Box 1. The emerging infectious diseases (EIDs) of wild birds discussed in this review (ordered chronologically).

Plasmodium relictum: This protist is one of the causal agents of avian malaria and is among the earliest documented EIDs known to significantly affect wild birds. Following the accidental introduction of its mosquito vector, *Culex quinquefasciatus*, to the Hawaiian islands in the early 20th century, this novel disease devastated local populations of honeycreepers including the Apapane (*Himatione sanguinea*), Hawaii Amakihi (*Hemignathus virens*) and Iiwi (*Vestiaria coccinea*) and contributed to the extinction of several others. As a result, many native Hawaiian birds could only be found in large numbers in high elevation forests and islands that were free of mosquitos (Vanriper *et al.* 1986; Warner 1968). However, based on mist-netting surveys conducted on the island of Hawaii in 2002, Hawaii Amakihi have persisted and increased in abundance at low elevations where *P. relictum* is prevalent, such that Hawaii Amakihi are more abundant at low elevations than at high elevations (Woodworth *et al.* 2005). Furthermore, these populations have been shown to be genetically isolated from high elevation populations (Foster *et al.* 2007), creating a unique system in which the evolution of host immunity to EIDs can be examined (Atkinson *et al.* 2013).

Mycoplasma gallisepticum: In 1994, the poultry pathogen *M. gallisepticum* was found to be the causative agent of a novel conjunctivitis disease observed in house finches (*Haemorhous mexicanus*) in Maryland, United States. Within 3–4 years, this bacterial pathogen spread throughout the entire eastern range of the house finch in North America killing an estimated tens of millions of house finches. These deaths resulted in part from the manifestation of *M.*

gallisepticum as a respiratory disease as well and in part from the conjunctivitis-induced blindness leading to starvation and increased susceptibility to predation (Dhondt *et al.* 2005; Fischer *et al.* 1997; Hartup *et al.* 2001a; Ley *et al.* 1996; Nolan *et al.* 1998).

West Nile Virus: In 1999, a novel highly pathogenic strain of West Nile Virus was found to be responsible for the unusually high numbers of bird deaths in New York (NY), United States (Bernard *et al.* 2001; Eidson *et al.* 2001a; Eidson *et al.* 2001b; Kramer & Bernard 2001b). While some affected birds displayed no symptoms before death, the most affected species such as American crows (*Corvus brachyrhynchos*) displayed severe symptoms including anorexia, weakness and mass loss as well as neurological problems such as ataxia, tremors, circling, disorientation and impaired vision resulting from WNV-induced encephalitis (Brault *et al.* 2004). Sequence analysis of WNV isolates from this epidemic found NY-1999 WNV isolates to be most closely related to WNV isolated from a dead goose in Israel in 1998 (Lanciotti *et al.* 1999). Combined with a lack of evidence for WNV in the United States before 1999, the epidemic was likely the result of a novel introduction of WNV to the United States with a probable Mediterranean origin (Kramer & Bernard 2001a, b; Lanciotti *et al.* 1999).

Highly pathogenic avian influenza (HPAI) virus: Historically waterfowl have been considered asymptomatic carriers of avian influenza viruses. However, H5N1 HPAI was found to be responsible for the deaths of new and old world species of ducks, geese and swans in two Hong Kong parks between December 2002 and January 2003. Affected birds exhibited symptoms ranging from slight inactivity, inappetence and ruffled feathers to severe neurological symptoms including paresis, paralysis, tremors and unusual head tilt, with death often occurring within 24 h

of the onset of symptoms (Ellis *et al.* 2004). Indeed, H5N1 isolates collected during the outbreak were found to cause systemic disease and similar severe clinical symptoms in mallards (*Anas platyrhychos*), whereas 1997 and 2001 H5N1 isolates from Hong Kong did not (Sturm-Ramirez *et al.* 2004). Subsequent outbreaks of HPAI affecting waterfowl occurred in China (Chen *et al.* 2005; Liu *et al.* 2005), Japan (Sakoda *et al.* 2012) and Bangladesh (Khan *et al.* 2014) as well as numerous European countries in 2006 (Smietanka *et al.* 2010; Weber *et al.* 2007).

Trichomonas gallinae: In 2005, a clonal strain of the protozoan *T. gallinae* spread from wild columbiform birds to chaffinches (*Fringilla coelebs*) and greenfinches (*Carduelis chloris*) in Great Britain, causing the loss of an estimated half a million birds by 2007 (Robinson *et al.* 2010). While chaffinch populations began to stabilize, greenfinch populations further declined, with an estimated population decrease from 4.3 to 2.8 million, or overall 1.5 million, greenfinches by 2009 (Lawson *et al.* 2012). Since then, *T. gallinae* has spread to finches in other European countries including Norway, Sweden and Finland (Neimanis *et al.* 2010) and has been found to cause disease in raptors including sparrowhawks (*Accipiter nusis*) and tawny owls (*Strix aluco*), presumably due to consumption of infected finches (Chi *et al.* 2013).

Figures

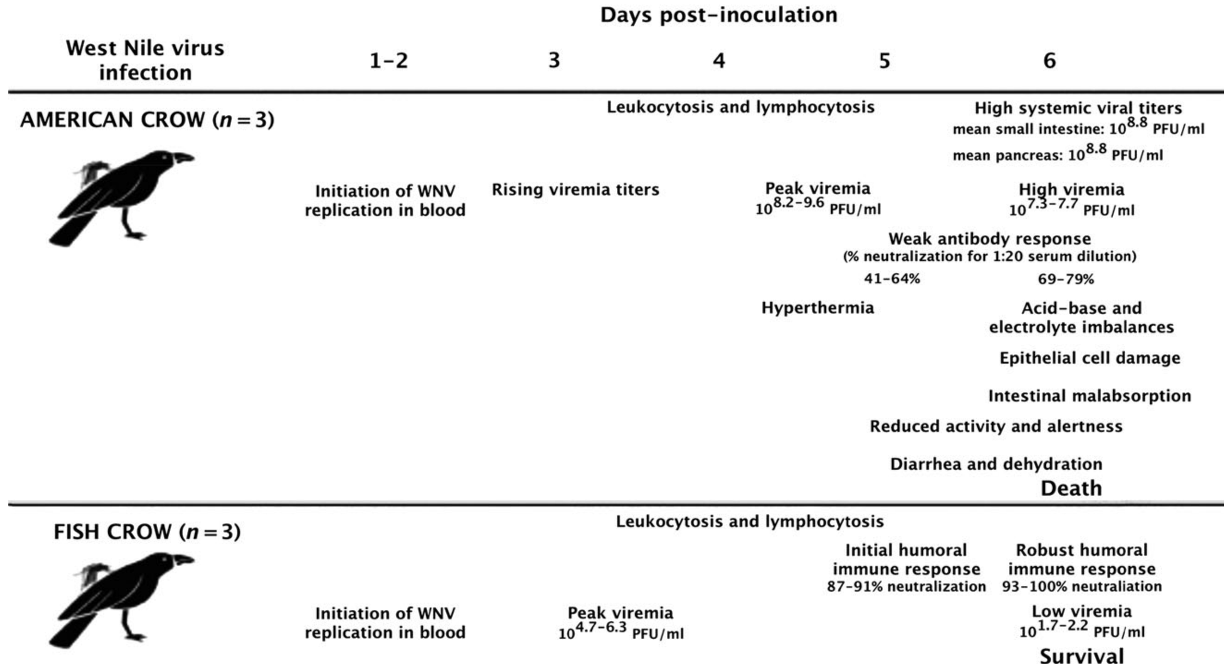

Figure 1. Using experimental WNV infections in American crows (*Corvus brachyrnchos*) and fish crows (*Corvus ossifragus*), Nemeth and colleagues show differences in disease progression and outcome between these two species that may be associated with differences in humoral immune responses (Modified from (Nemeth *et al.* 2011)).

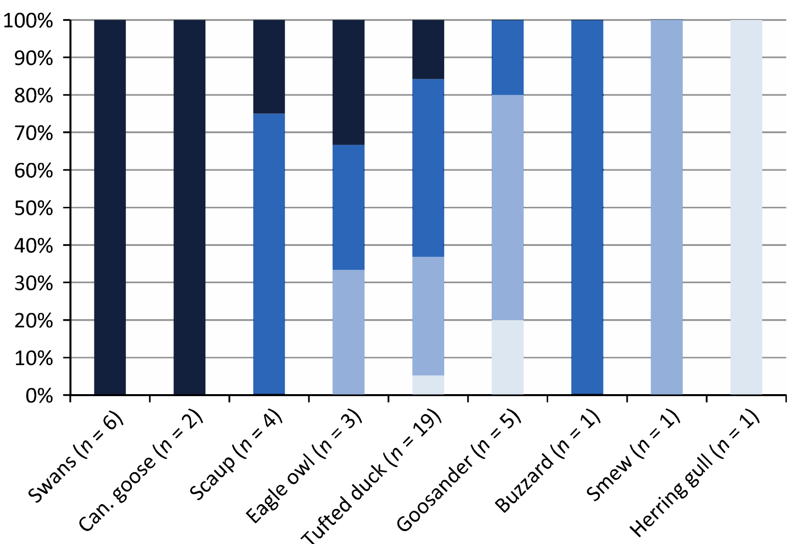

Figure 2. Severity of H5N1 HPAI encephalitis in nine naturally infected wild bird species: mute swans (*Cygnus olor*), Canada geese (*Branta Canadensis*), greater scaup (*Aythya marila*), European eagle owls (*Bubo bubo*), tufted duck (*Aythya fuligula*), goosander (common merganser; *Mergus merganser*), common buzzard (*Buteo buteo*), smew (*Mergellus albellus*) and herring gull (*Larus argentatus*). Severity is based on use of immunohistochemistry to assess intensity and area of staining for the following: total area of inflammation, inflammatory components, viral antigen prevalence, neuronal changes and vascular changes (From (Brojer *et al.* 2012)).

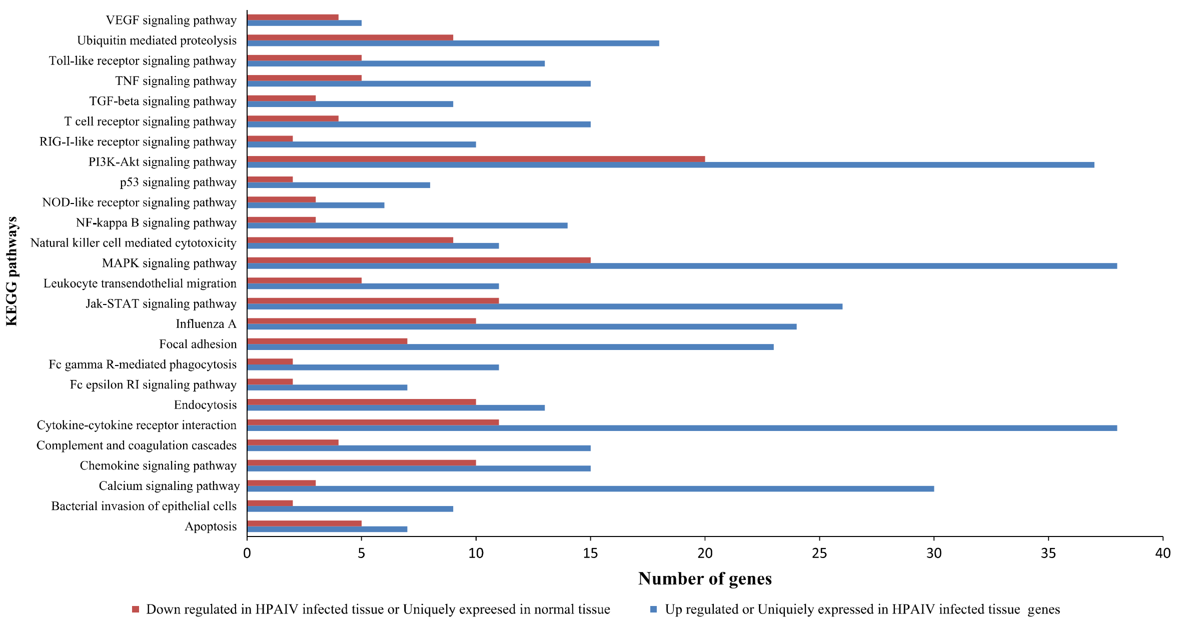

Figure 3. KEGG pathway analysis of differentially expressed genes in the lungs of non-infected vs. HPAI-infected jungle crows (*Corvus macrorhynchos*) (From (Vijayakumar *et al*. 2015)).

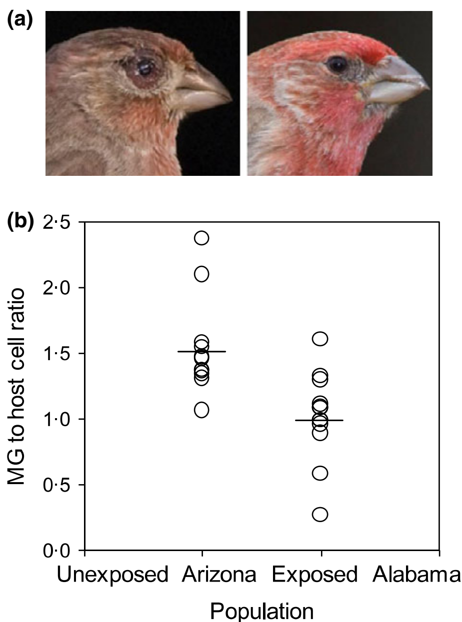

Figure 4. Symptoms of *Mycoplasma gallisepticum* infection and pathogen load in the conjunctivae of house finches. (a) Symptoms of *Mycoplasma gallisepticum* infection in naturally infected (left) and healthy (right) wild house finches. (b) Quantification of bacterial load in the conjunctiva of infected finches from disease-exposed and unexposed populations, 2 weeks post-infection (From (Bonneaud *et al*. 2011)).

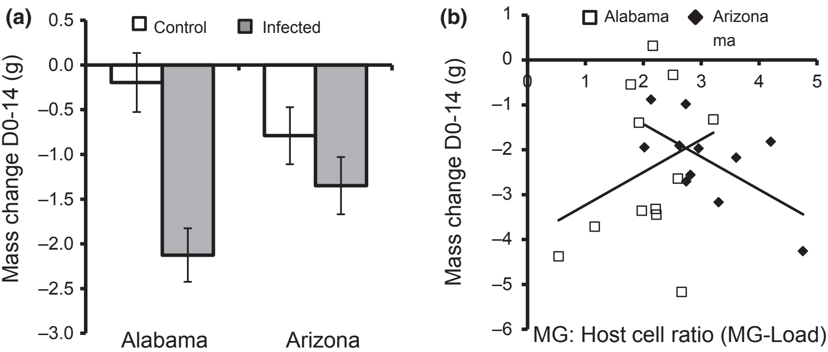

Figure 5. Mass loss in *Mycoplasma gallisepticum*-infected house finches vs. sham-inoculated controls and bacterial load in the conjunctivae of infected finches. (a) Effects of infection with *Mycoplasma gallisepticum* vs. sham inoculations on mass change (g) between days 0 and 14 post-infection in finches from disease-exposed (Alabama) and disease-unexposed (Arizona) populations. (b) Association between bacterial load 14 days post-infection and mass change (g) between days 0 and 14 post-infection in birds from Alabama (open squares) and Arizona (filled diamonds) (From (Bonneaud *et al.* 2012a)).

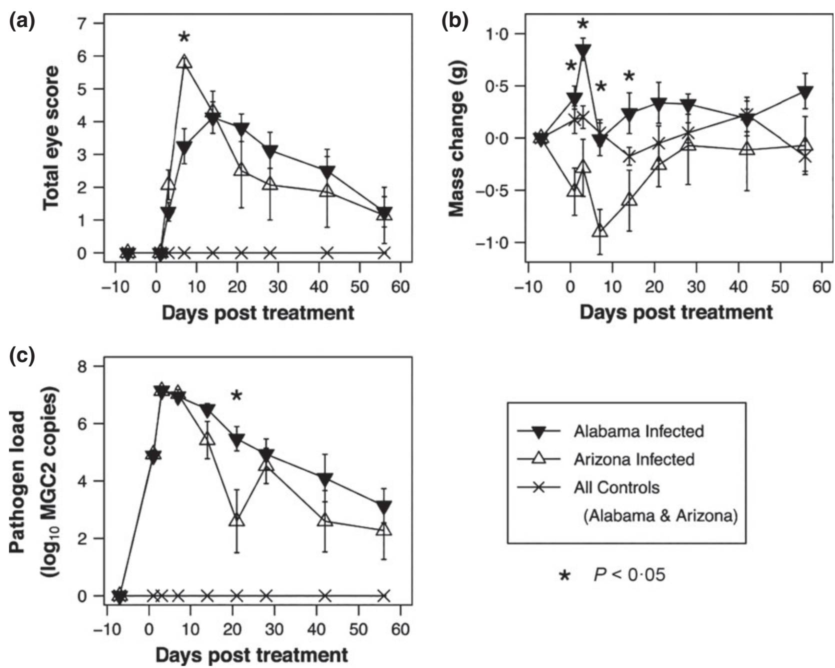

Figure 6. Pathology of house finches infected with *Mycoplasma gallisepticum* and originating either from disease-exposed (Alabama) or disease-unexposed (Arizona) populations. Finches from the exposed population displayed lower peak eye lesion score (a) and reduced mass loss (b) relative to finches from unexposed populations, despite similar bacterial load (c) (From (Adelman *et al.* 2013)).

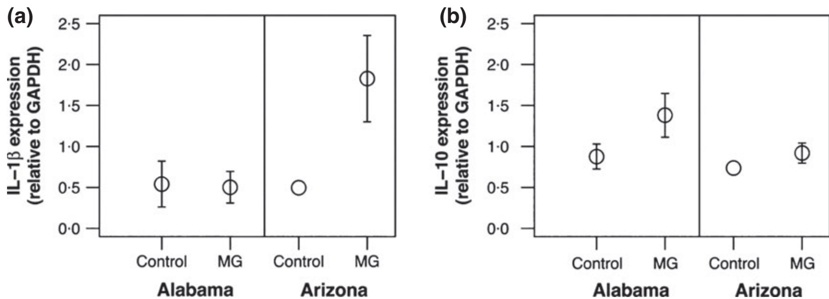

Figure 7. Expression of the inflammatory cytokines in the blood of house finches infected with *Mycoplasma gallisepticum* and originating either from disease-exposed (Alabama) or disease-unexposed (Arizona) populations. Expression of the pro-inflammatory cytokine IL-1b was significantly lower in finches from the exposed population (a), but expression of the anti-inflammatory cytokine IL-10 was marginally higher in those individuals relative to those from the unexposed population (b) (From (Adelman *et al.* 2013)).

CHAPTER 2

Occurrence of *Mycoplasma gallisepticum* in house finches (*Haemorhous mexicanus*) from Arizona, USA

Abstract

In 1994, an endemic poultry pathogen, *Mycoplasma gallisepticum* (*Mg*), was identified as the causative agent of a novel disease in house finches (*Haemorhous mexicanus)*. Following an initial outbreak in Maryland, *Mg* rapidly spread in house finches throughout eastern North America, killing millions of birds. Subsequently, *Mg* spread slowly through the northern interior of North America and then into the Pacific Northwest, finally reaching California in 2006. Through 2010, there were no reports of *Mg* in the southwestern United States east of California. In August 2011, following reports of house finches displaying conjunctivitis symptoms characteristic of *Mg* infection in Arizona, we trapped house finches at bird feeders in central Arizona (Tempe) and southern Arizona (Tucson and Green Valley) to assay for *Mg* infection. Upon capture, we noted whether birds exhibited conjunctivitis symptoms and we collected choanal swabs to test for the presence of *Mg* DNA using PCR. We detected *Mg* in finches captured from Green Valley (in ~12% of birds captured) but not from Tucson or Tempe. Based on resampling of house finches at these sites in July 2014, central Arizona finches likely remain unexposed to *Mg*. We suggest that low urban connectivity between arid habitats of southern and central Arizona or a reduction in the prevalence of *Mg* following its initial arrival in Arizona may

be limiting the spread of *Mg* from south to north in Arizona. We further suggest that the observed conjunctivitis-like symptoms in house finches that were negative for *Mg* by PCR may be caused primarily by avian pox.

Introduction

The colonization of a novel host by a pathogen offers the opportunity to study the conditions under which host shifts occur, the mode and tempo of transmission through the novel host population, and changes in host-pathogen dynamics through time (e.g.(Adelman *et al.* 2013b; Bonneaud *et al.* 2011; Dhondt *et al.* 2006; Hochachka & Dhondt 2000)). For such studies to be most revealing, accurate surveys of pathogen prevalence throughout the range of the host, particularly near the leading edge of pathogen spread, are critical.

One of the best studied host shifts in recent decades is the infection of North American house finches (*Haemorhous mexicanus*) by the bacterial pathogen *Mycoplasma gallisepticum* (*Mg*). *Mg* was first detected in wild house finches in 1994 in Maryland after an apparent jump from poultry (Delaney *et al.* 2012; Fischer *et al.* 1997; Ley *et al.* 1996). Following its initial appearance, *Mg* spread rapidly through eastern North American house finch populations. The spread of this novel disease was unusually well-documented due to the visible conjunctivitis symptoms caused by *Mg* as well as active disease monitoring by biologists and through the House Finch Disease Survey organized by the Cornell University Laboratory of Ornithology (Dhondt *et al.* 1998; Fischer *et al.* 1997; Hartup *et al.* 1998; Luttrell *et al.* 1996; Roberts *et al.* 2001b). Within a year of the outbreak, *Mg* had spread beyond the Mid-Atlantic States and by 1995 had reached all of New England and as far south as Georgia. By 1997, house finches with conjunctivitis had been reported in all states east of the Rocky Mountains (Dhondt *et al.* 1998;

Fischer *et al.* 1997) (Figure 1). As *Mg* spread through these house finch populations, it killed millions of birds (Nolan *et al.* 1998), reducing the entire eastern North American house finch population to about half of its pre-epizootic size (Hochachka & Dhondt 2000).

In contrast to the rapid spread of *Mg* throughout the eastern states, *Mg* has spread more slowly through western North America. In addition, western *Mg* cases have been more difficult to verify because of the higher prevalence of avian poxvirus in western house finch populations, which like *Mg* can cause swelling around the eyes (Dhondt *et al.* 2006). Previous studies have estimated that avian pox infections are present in approximately 20% of western house finches versus less than 5% of eastern house finches (Davis *et al.* 2013). However, *Mg* infection of house finches west of the Rocky Mountains was confirmed through polymerase chain reaction (PCR)-based testing of house finches caught in Montana in 2002. Subsequently, DNA and culture-based evidence confirmed the presence of *Mg* in the Pacific Northwest and California in the early to mid 2000s (Figure 1) (Dhondt *et al.* 2006; Duckworth *et al.* 2003; Hawley *et al.* 2010; Ley *et al.* 2006). However, at no point over this time have reports of *Mg* in the arid southwest regions of the United States east of California been confirmed by direct sampling. Here we present results of house finch sampling in which we assessed clinical *Mg* symptoms and screened house finches for *Mg* by PCR in two areas of Arizona (southern and central portions of the state), presumably at the leading edge of the *Mg* spread.

Materials and methods

House finch sampling

Following reports on house finch mortality associated with conjunctivitis symptoms in late 2009-early 2010 in Arizona (Badyaev *et al.* 2012), in August of 2011 we sampled house

finches in Arizona to assess the occurrence of *Mg* in house finch populations. Our goal was to sample the Arizona Sun Corridor mega-region (Phoenix and Tucson metropolitan areas) to determine whether MG was present and the geographic extent of the disease. Birds with *Mg*-like symptoms were reported in Tucson and Green Valley (Badyaev *et al.* 2012), whereas regular monitoring since 2004 of house finches in the Phoenix area produced no reports of *Mg*-like conjunctivitis (McGraw, pers. obs.). We subsequently resampled these populations in July 2014 to examine potential temporal changes in disease prevalence and distribution.

In August 2011, we trapped house finches at bird feeding stations in southern Arizona (Tucson and Green Valley; 32.46 N, 110.94 W and 31.87 N, 110.96 W, respectively) and central Arizona (Tempe/Phoenix; 33.42 N, 111.93 W), USA. We trapped 21, 69, and 35 house finches in Tucson, Green Valley, and Tempe (Phoenix), respectively (Hill 2002). Tucson is approximately 180 km southeast of Tempe and 50 km north of Green Valley (Figure 2). In July 2014, we sampled 50 finches from Tempe, 66 from Tucson, 32 from Green Valley, and 61 birds from two additional southern Arizona locations near Amado (which is ca. 20 km south of Green Valley; 31.66 N, 111.23 W and 31.75 N, 111.00 W; Figure 2).

Upon capture, a choanal swab was collected from each bird to test for the presence of *Mg* (Roberts et al. 2001). Additionally, birds were assessed for conjunctivitis-like symptoms (i.e. eye swelling) associated with *Mg* infection (Farmer *et al.* 2002). Due to a lack of observed conjunctivitis symptoms in 2014, we collected blood from a subset of 50 individuals in Tucson and tested the serum for *Mg* antibodies by serum plate agglutination using a commercially available *Mg* Plate Antigen (Charles River Laboratories, 10100760) (Roberts *et al.* 2001a).

Mg presence

Choanal swabs were tested for the presence of *Mg* via PCR (Roberts *et al.* 2001a). Briefly, swabs were placed in 100 µl of sterile nuclease free water. Swabs were then placed at 100 °C for 10 minutes, at -20 °C for 10 minutes, and finally centrifuged at 13000 rpm for 5 minutes. We tested the supernatant of each sample in duplicate for *Mg* presence using the forward primer 5' GCTTCCTTGCGGTTAGCAAC 3' and reverse primer 5' GAGCTAATCTGTAAAGTTGGTC 3'. PCR parameters were as follows: 94° C for 5 minutes, 35 cycles of 94° C for 30 seconds, 55° C for 30 seconds, and 72° C for 30 seconds, and a final 5 minute extension at 72° C (Roberts *et al.* 2001a). In each assay, *Mg* DNA extracted from pure culture served as a positive control.

Results

In 2011, 10 of 69 house finches from Green Valley and 2 of 21 birds from Tucson had conjunctivitis-like symptoms. Of these 12 birds, only seven individuals, all from Green Valley, were confirmed positive for *Mg* via PCR testing of the choanal swabs. Additionally, one asymptomatic bird from Green Valley tested positive for *Mg* via PCR, yielding 8 of 69 (11.6%) infected birds captured in Green Valley (Table 1). In contrast, in 2014 no birds sampled from Tucson, Green Valley, or Amado were symptomatic or PCR positive for *Mg* based on choanal swabs. Of the 50 individuals from Tucson whose serum was tested for *Mg* antibodies by rapid plate agglutination, only five (10%) were found positive. No individuals were found to have conjunctivitis symptoms in Tempe in 2011, while three birds exhibited suspect conjunctivitis-like symptoms in 2014. However, all birds sampled in Tempe tested negative for *Mg* by PCR

(Table 1). Thus, no cases of *Mg* infection have been confirmed in the Phoenix-Tempe metropolitan area to date.

Discussion

Our study confirms the occurrence of *Mg* in southern Arizona by 2011. Although we did not sample birds from these populations prior to 2011 or at neighboring sites (i.e. along a disease-transmission path), it is possible that *Mg* arrived at Green Valley via the leading edge of its spreading range from the east rather than from California in the west. By contrast, finches from central Arizona—specifically the Tempe/Phoenix region—appear to remain unexposed to *Mg* as of 2014.

The absence of *Mg* in house finches from Tempe, which is about 180 km north of a site where *Mg* is known to be present (Green Valley), could be due to the lack of urban habitat between house finch populations in this arid region (i.e. along the Arizona Sun Corridor) and hence reduced opportunity for transmission from south to north. Transmission of *Mg* between individuals requires direct exposure to moisture droplets containing *Mg* (Dhondt *et al.* 2005). Shared, anthropogenic resources such as bird feeders that bring sick and healthy individuals into contact were thought to have played a major role in *Mg*'s initial epizootic spread (Adelman *et al.* 2013a; Dhondt *et al.* 2007), in part due to facilitating higher host abundance (Hosseini *et al.* 2006). However, despite high abundance of house finches throughout their native western range, in some populations, particularly Sonoran Desert populations in California and Arizona, there is no or only short-distance (less than 10 km) migration of house finches (though there is potential for contact between populations via juvenile dispersal; (Badyaev *et al.* 2012; Badyaev *et al.* 2008). For instance, house finches from geographically adjacent urban (University of Arizona,

Tucson) and undisturbed natural Sonoran Desert (Saguaro West National Park) habitats display divergence in traits related to bill development and bite force (Badyaev *et al.* 2008). The divergence between these populations, separated by only 25 km, has been attributed to a lack of gene flow between populations combined with selection resulting from differences in available food resources due to human provisioning (Badyaev *et al.* 2008). Based on 12 microsatellite loci, these same populations exhibit numerically small ($F_{ST} = 0.003$) but highly statistically significant genetic differentiation at levels typical of house finch populations separated by much greater distances (>800 km) in other parts of North America (Badyaev *et al.* 2008). Therefore, if we also assume lack of gene flow between Green Valley and Tempe birds, *Mg* may not be able to spread to central Arizona house finches from the south.

Alternatively, the failure of *Mg* to infect the Tempe population may be a consequence of the virulence of the *Mg* isolates currently circulating in Arizona. Indeed, models indicate that the virulence of *Mg* isolates at the disease front are likely to be lower than those found in regions where *Mg* has become endemic and thus potentially would have reduced transmissibility (Osnas *et al.* 2015). In agreement with these models, a common garden experiment using house finches and *Mg* isolates from Virginia and California found that a 2006 *Mg* isolate from California had lower virulence than a 1994 Virginia *Mg* isolate (Hawley *et al.* 2010). Similarly, this same 2006 California *Mg* isolate exhibited significantly lower virulence and transmissibility in house finches from eastern populations (Williams *et al.* 2014), which presumably have evolved *Mg* resistance mechanisms (Bonneaud *et al.* 2011), than a North Carolina 2006 isolate. While of lower virulence, this California isolate exhibited comparable transmissibility to the Virginia 1994 *Mg* isolate (Williams *et al.* 2014). However, by 2010 *Mg* isolates circulating in California had demonstrably increased in virulence to a level comparable to the Virginia 1994 *Mg* isolate

(Hawley *et al.* 2013). Given that, in August 2011 (two years after *Mg* was initially reported in Arizona), approximately 12% of house finches in Green Valley were found to be infected with *Mg*, it would suggest that reduced *Mg* virulence was not a major factor inhibiting *Mg*'s spread to central Arizona.

Contrary to what would be expected given the observed incidence of *Mg* in Green Valley in 2011, we trapped no birds with active *Mg* infections at the same site in July 2014. Unlike the low occurrence of *Mg* in May-July seen in eastern and Montana house finch populations (Altizer *et al.* 2004; Dhondt *et al.* 2006), in Arizona *Mg* is thought to be most prevalent during spring and summer months, with reduced prevalence in fall and winter (Badyaev *et al.* 2012). Thus, possible explanations for change in prevalence of *Mg* include 1) annual fluctuations in *Mg* infections, 2) *Mg* is no longer present in house finches from Green Valley, or 3) limited sampling. Just as low pathogen virulence may inhibit disease transmission, too high of pathogen virulence may also inhibit transmission (Park *et al.* 2013). Based on initial reporting on the arrival of *Mg* to Arizona in 2009-2010, *Mg* infections induced up to 70% local adult house finch mortality (Badyaev *et al.* 2012). If infected birds died without transmitting the disease, *Mg* prevalence may have rapidly diminished in these populations (Park *et al.* 2013). To elucidate the underlying reason for the apparent absence of *Mg* in Arizona in July 2014, continued and more thorough monitoring of southern Arizona house finch populations is necessary.

Although we have confirmed the presence of *Mg* in southern Arizona, it is important to note that additional birds in all sampling locations were found to have some degree of eye swelling that was not attributable to *Mg* (i.e. they tested negative for *Mg* DNA). The difference in clinical presentation of symptoms in these birds versus known *Mg*-infected birds (Figure 3) suggests that another disease, such as avian pox, may be responsible for the observed symptoms.

Avian poxviruses can cause swollen lesions on the eyes (in addition to other areas of the body) and thus be mistaken for *Mg* -associated conjunctivitis (Dhondt *et al.* 2006; Parker *et al.* 2011; Weli & Tryland 2011). However, unlike *Mg* such lesions are not associated with a watery discharge. Rather, pox lesions typically have a dry or crusty appearance, and are occasionally bloody, as transmission of avian poxvirus occurs predominantly through insect vectors and skin abrasions. Additionally, pox lesions that occur on/around the eye are typically localized to a particular ocular region, whereas during an *Mg* infection the entire conjunctiva tends to become inflamed (Dhondt *et al.* 2005; Weli & Tryland 2011). From 2000-2002, before *Mg* was detected in the Pacific Northwest and long before *Mg* reached California or the southwestern United States, house finches with conjunctivitis-like symptoms were reported in all of these regions through the House Finch Disease Survey and Project Feederwatch (Dhondt *et al.* 2006). Following *Mg*'s arrival in Montana, reports of conjunctivitis increased drastically, suggesting that the previously reported instances of conjunctivitis-like symptoms may have been misidentified as *Mg* (Dhondt *et al.* 2006). While our sampling methods were insufficient for a definitive diagnosis, incorporation of protocols for testing for avian poxviruses (Gyuranecz *et al.* 2013; Manarolla *et al.* 2010) or other possible pathogens could help estimate the proportion of reported conjunctivitis cases that can be attributed to *Mg* versus avian pox and thus help clarify the pattern of *Mg* occurrence across western North America.

In conclusion, our findings confirm the presence of *Mg* in southern Arizona and provide further detail regarding the occurrence and potential spread of *Mg* in the southwestern United States. Furthermore, our data support the persistence of a *Mg* -unexposed population of house finches in central Arizona. These populations can continue to be an invaluable resource for studying host-pathogen co-evolution in this system.

Acknowledgements

This study was funded by NSF grants (DEB1113666and IOS0923600) and a Marie Curie Reintegration grant (FP7-PEOPLE-IRG-2008 #239257). Additionally, Staley was supported by an Auburn University Cellular and Molecular Biosciences Peaks of Excellence Graduate Research Fellowship. Procedures conducted in this study were approved by the Auburn University IACUC under PRNs 2010-1762 and 2014-2489. We thank D. Vleck, D. Cowan, J. Pestle, S. Masters, C. Howell, L. Camacho, A. Wyer, and L. Wolf for their assistance with this project. We also thank A. Badyaev for feedback on this manuscript.

References

Adelman JS, Carter AW, Hopkins WA, Hawley DM (2013a) Deposition of pathogenic *Mycoplasma gallisepticum* onto bird feeders: host pathology is more important than temperature-driven increases in food intake. *Biol Lett* **9**, 20130594.

Adelman JS, Kirkpatrick L, Grodio JL, Hawley DM (2013b) House finch populations differ in early inflammatory signaling and pathogen tolerance at the peak of *Mycoplasma gallisepticum* infection. *American Naturalist* **181**, 674-689.

Altizer S, Hochachka WM, Dhondt AA (2004) Seasonal dynamics of mycoplasmal conjunctivitis in eastern North American house finches Journal of Animal Ecology Volume 73, Issue 2. In: *Journal of Animal Ecology*, pp. 309-322.

Badyaev AV, Belloni V, Hill GE (2012) House finch (*Haemorhous mexicanus*). Ithaca: Cornell Lab of Ornithology.

Badyaev AV, Young RL, Oh KP, Addison C (2008) Evolution on a local scale: developmental, functional, and genetic bases of divergence in bill form and associated changes in song structure between adjacent habitats. *Evolution* **62**, 1951-1964.

Bonneaud C, Balenger S, Russell AF*, et al.* (2011) Rapid evolution of disease resistance is accompanied by functional changes in gene expression in a wild bird. *Proc Natl Acad Sci U S A* **108**, 7866-7871.

Davis AK, Hood WR, Hill GE (2013) Prevalence of blood parasites in eastern versus western house finches: are eastern birds resistant to infection? *Ecohealth* **10**, 290-297.

Delaney NF, Balenger S, Bonneaud C*, et al.* (2012) Ultrafast evolution and loss of CRISPRs following a host shift in a novel wildlife pathogen, *Mycoplasma gallisepticum*. *PLoS Genet* **8**, e1002511.

Dhondt AA, Altizer S, Cooch EG*, et al.* (2005) Dynamics of a novel pathogen in an avian host: Mycoplasmal conjunctivitis in house finches. *Acta Trop* **94**, 77-93.

Dhondt AA, Badyaev AV, Dobson AP*, et al.* (2006) Dynamics of mycoplasmal conjunctivitis in the native and introduced range of the host. *Ecohealth* **3**, 95-102.

Dhondt AA, Dhondt KV, Hawley DM, Jennelle CS (2007) Experimental evidence for transmission of *Mycoplasma gallisepticum* in house finches by fomites. *Avian Pathol* **36**, 205-208.

Dhondt AA, Tessaglia DL, Slothower RL (1998) Epidemic mycoplasmal conjunctivitis in house finches from Eastern North America. *J Wildl Dis* **34**, 265-280.

Duckworth RA, Badyaev AV, Farmer KL, Hill GE, Roberts SR (2003) First case of *Mycoplasma gallisepticum* infection in the western range of the house finch (*Carpodacus mexicanus*). *Auk* **120**, 528-530.

Farmer KL, Hill GE, Roberts SR (2002) Susceptibility of a naive population of house finches to Mycoplasma gallisepticum. *J Wildl Dis* **38**, 282-286.

Fischer JR, Stallknecht DE, Luttrell MP, Dhondt AA, Converse KA (1997) Mycoplasmal conjunctivitis in wild songbirds: the spread of a new contagious disease in a mobile host population. *Emerg Infect Dis* **3**, 69-72.

Gyuranecz M, Foster JT, Dan A*, et al.* (2013) Worldwide phylogenetic relationship of avian poxviruses. *J Virol* **87**, 4938-4951.

Hartup BK, Mohammed HO, Kollias GV, Dhondt AA (1998) Risk factors associated with mycoplasmal conjunctivitis in house finches. *J Wildl Dis* **34**, 281-288.

Hawley DM, Dhondt KV, Dobson AP*, et al.* (2010) Common garden experiment reveals pathogen isolate but no host genetic diversity effect on the dynamics of an emerging wildlife disease. *J Evol Biol* **23**, 1680-1688.

Hawley DM, Osnas EE, Dobson AP*, et al.* (2013) Parallel patterns of increased virulence in a recently emerged wildlife pathogen. *PLoS Biol* **11**, e1001570.

Hill GE (2002) *A red bird in a brown bag : the function and evolution of colorful plumage in the House Finch* Oxford University Press, Oxford ; New York.

Hochachka WM, Dhondt AA (2000) Density-dependent decline of host abundance resulting from a new infectious disease. *Proc Natl Acad Sci U S A* **97**, 5303-5306.

Hosseini PR, Dhondt AA, Dobson AP (2006) Spatial spread of an emerging infectious disease: conjunctivitis in house finches. *Ecology* **87**, 3037-3046.

Ley DH, Berkhoff JE, McLaren JM (1996) *Mycoplasma gallisepticum* isolated from house finches (*Carpodacus mexicanus*) with conjunctivitis. *Avian Dis* **40**, 480-483.

Ley DH, Sheaffer DS, Dhondt AA (2006) Further western spread of Mycoplasma gallisepticum infection of house finches. *J Wildl Dis* **42**, 429-431.

Luttrell MP, Fischer JR, Stallknecht DE, Kleven SH (1996) Field investigation of *Mycoplasma gallisepticum* infections in house finches (*Carpodacus mexicanus*) from Maryland and Georgia. *Avian Dis* **40**, 335-341.

Manarolla G, Pisoni G, Sironi G, Rampin T (2010) Molecular biological characterization of avian poxvirus strains isolated from different avian species. *Vet Microbiol* **140**, 1-8.

Nolan PM, Hill GE, Stoehr AM (1998) Sex, size, and plumage redness predict house finch survival in an epidemic. *Proceedings of the Royal Society B-Biological Sciences* **265**, 961-965.

Osnas EE, Hurtado PJ, Dobson AP (2015) Evolution of pathogen virulence across space during an epidemic. *Am Nat* **185**, 332-342.

Park M, Loverdo C, Schreiber SJ, Lloyd-Smith JO (2013) Multiple scales of selection influence the evolutionary emergence of novel pathogens. *Philos Trans R Soc Lond B Biol Sci* **368**, 20120333.

Parker PG, Buckles EL, Farrington H*, et al.* (2011) 110 years of Avipoxvirus in the Galapagos Islands. *PLoS One* **6**, e15989.

Roberts SR, Nolan PM, Hill GE (2001a) Characterization of *Mycoplasma gallisepticum* infection in captive house finches (*Carpodacus mexicanus*) in 1998. *Avian Dis* **45**, 70-75.

Roberts SR, Nolan PM, Lauerman LH, Li LQ, Hill GE (2001b) Characterization of the mycoplasmal conjunctivitis epizootic in a house finch population in the southeastern USA. *J Wildl Dis* **37**, 82-88.

Weli SC, Tryland M (2011) Avipoxviruses: infection biology and their use as vaccine vectors. *Virol J* **8**, 49.

Williams PD, Dobson AP, Dhondt KV, Hawley DM, Dhondt AA (2014) Evidence of trade-offs shaping virulence evolution in an emerging wildlife pathogen. *J Evol Biol* **27**, 1271-1278.

Tables

Table 1. Results from 2011 and 2014 house finch sampling in southern and central Arizona.

Year	Sampling Locations	Conjunctivitis-like symptoms	Number PCR positive	Number antibody positive
2011	**Southern Arizona**			
	Tucson	2/21	0/21	-
	Green Valley	10/69	8/69	-
	Central Arizona			
	Tempe (Phoenix)	0/35	0/35	-
2014	**Southern Arizona**			
	Tucson	0/32	0/32	-
	Green Valley	0/66	0/66	5/50
	Amado 1	0/25	0/25	-
	Amado 2	0/36	0/36	-
	Central Arizona			
	Tempe (Phoenix)	3/50	0/50	-

Figures

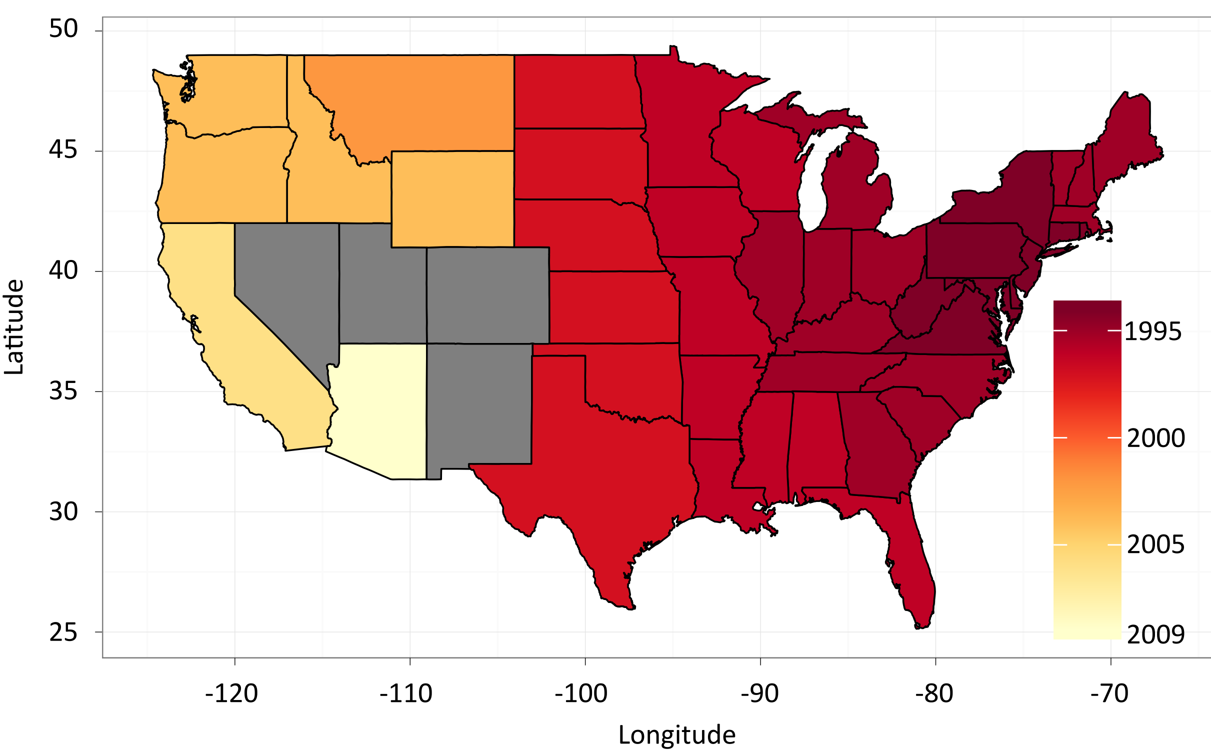

Figure 1. The reported spread of *Mycoplasma gallisepticum* throughout North America. Grey-shaded regions represent areas where MG occurrence is unknown (southwestern United States east of California).

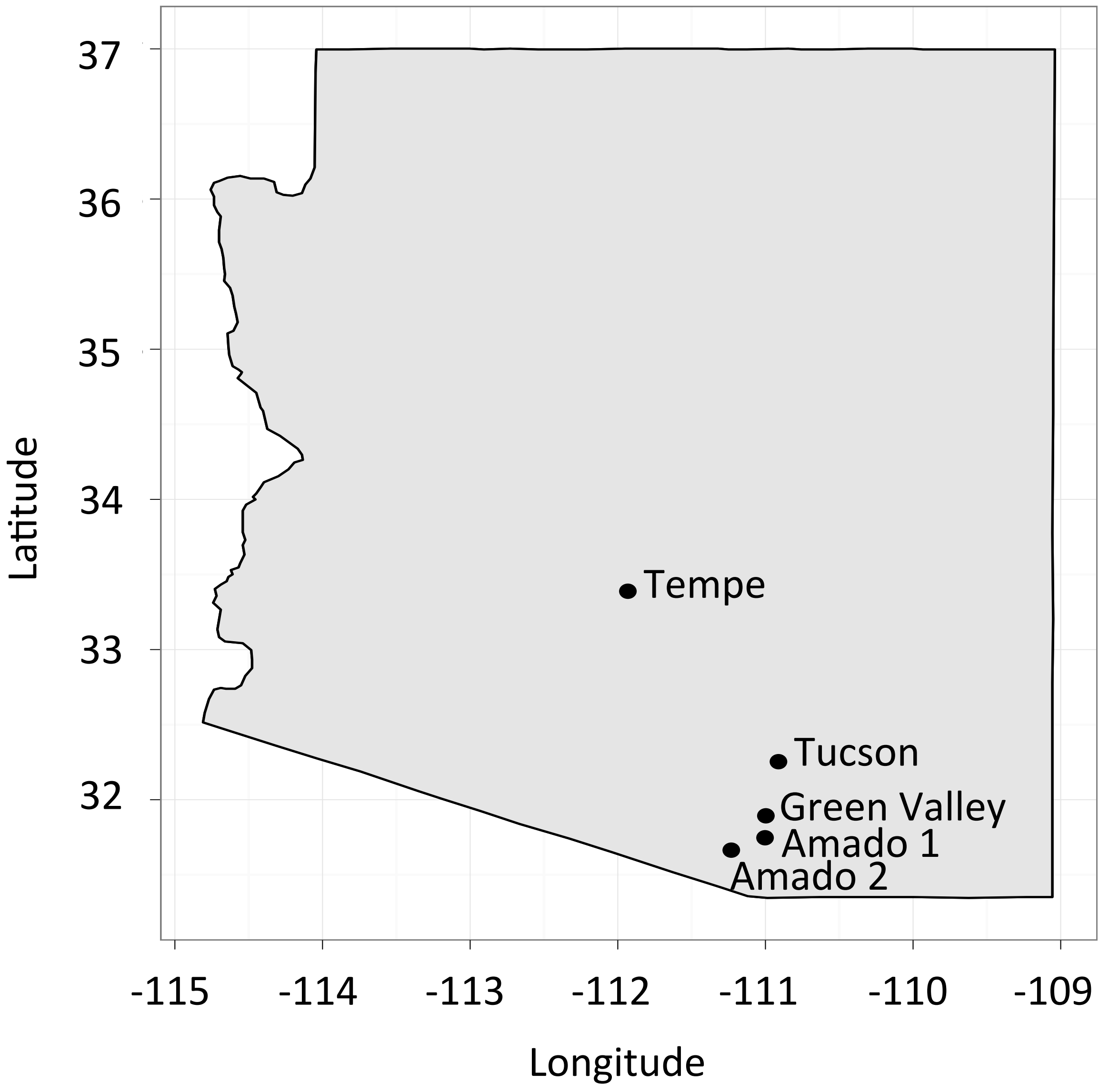

Figure 2. Central Arizona (Tempe) and southern Arizona (Tucson, Green Valley, Amado) sampling locations.

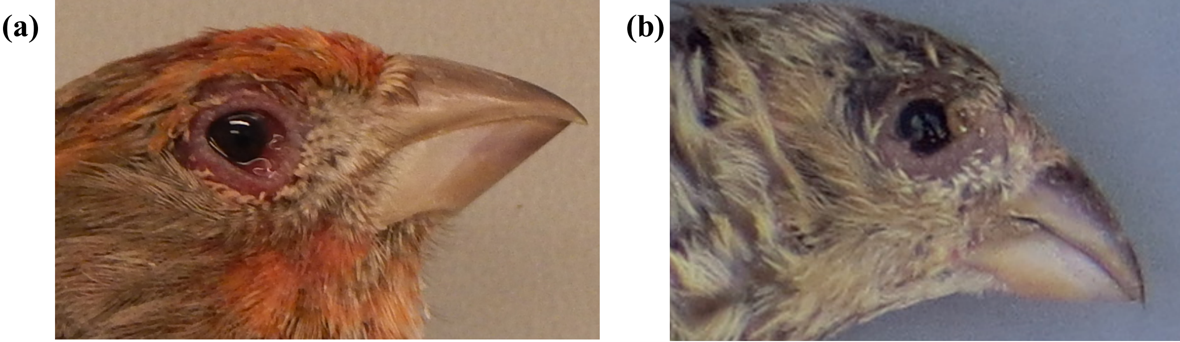

Figure 3. (a) House finch displaying a characteristic *Mycoplasma gallisepticum* infection, defined by swelling of the conjunctiva consistently around the eye and a watery discharge. (b) House finch trapped in Tempe, Arizona, with suspected avian poxvirus. Pox lesions begin as small wart-like lesions (<3 mm) and may become enlarged with a dry, crusty, and occasionally bloody appearance.

CHAPTER 3

Testing the role of host exposure in the host shift of an emerging bacterial pathogen

Abstract

The shift to a novel host by a pathogen requires both access to the new host and the capacity to survive and replicate in the novel host environment. To assess the role of host exposure in limiting bacterial host shifts, we studied the jump of the bacterium *Mycoplasma gallisepticum* (*Mg*) from domestic poultry into house finches (*Haemorhous mexicanus*). We experimentally inoculated house finches with strains of *Mg* obtained either from poultry (Rlow) or from house finches (1995 epizootic outbreak strain: HF1995). All 15 house finches inoculated with the HF1995 house finch *Mg* strain became infected and the infection persisted for an average of ~30 days. In contrast, Rlow only successfully colonized the mucosal epithelium of the upper respiratory tract in 12 of 15 (80%) inoculated house finches and persisted an average of only ~10 days. Furthermore, Rlow achieved lower bacterial loads in the respiratory mucosa relative to HF1995. While 14 of 15 (93%) house finches infected with HF1995 became symptomatic, Rlow caused clinical symptoms in only 5 (33%) individuals and these were significantly milder than in finches inoculated with HF1995. Given the reduced ability of Rlow to colonize and persist within house finches and to give rise to clinical symptoms, our results indicate that, while a prerequisite for host shifting, exposure was not the limiting factor in this

disease emergence event. Instead, adaptive genetic changes in *Mg* are likely to have been necessary for sustained pathogen transmission in the novel house finch population.

Introduction

Recent outbreaks of novel diseases in humans and domestic animals underscore the critical need to examine the factors that allow pathogens to jump into new hosts (Chen *et al.* 2005; Kramer & Bernard 2001; Ley *et al.* 1996; Liu *et al.* 2005; Viana *et al.* 2015). There are generally two key factors that determine whether a pathogen can undergo a host shift: exposure of the pathogen to a novel host and the pathogen's capacity to infect and be transmitted by the new host (Lambrechts 2010; Woolhouse *et al.* 2005). Exposure depends on bringing a pathogen, its original host, and the novel host together in time and space and will be mitigated by the ecologies of the two hosts and the pathogen (Leroy *et al.* 2005). Infectiousness and potential for transmission, in contrast, will primarily be determined by pathogen and host genotypes (Parrish *et al.* 2008; Taubenberger & Kash 2010). The contribution of ecological versus genetic factors in mediating pathogen emergence in novel hosts remains to be fully elucidated.

Testing whether host exposure may limit host shifting is a crucial first step to disentangling the contribution of ecological versus genetic factors. However, such experiments aimed at elucidating the role of host exposure can also provide insight into whether observed pathogen genetic changes were likely to be necessary for host shifting or, alternatively, a consequence of selection for persistence within and sustained transmission among the novel host (Engering *et al.* 2013; Park *et al.* 2013). For instance, the natural host of the myxoma virus is a South American leporid rabbit (*Sylvagus brasiliensis*), but this virus is also able to infect European rabbits (*Oryctolagus cuniculus*), to which it is not naturally exposed to, and was, as a result, used in an

eradication attempt of this host species in Australia mid-20th century. Despite exposure being limiting in this instance, subsequent monitoring revealed rapid selection on the myxoma virus for attenuated virulence, facilitating sustained transmission among European rabbits (Kerr 2012; Kerr *et al.* 2012). In the case of highly pathogenic avian influenza, although exposure is occurring, transmission efficiency among novel mammalian host species is often low, presumably due to avian influenza being poorly adapted to binding receptors on mammalian host cells (Imai & Kawaoka 2012; Imai *et al.* 2012). Indeed, the influenza subtype H5N1 is not effectively transmitted between ferrets (Maines *et al.* 2006; Yen *et al.* 2007), yet a reassortant virus comprised of a mutated H5 heamagglutinin, involved in receptor binding, and an otherwise H1N1 genetic background can transmit in this mammal species via respiratory droplets (Imai *et al.* 2012). While these viral studies above illustrate the roles of exposure and host-pathogen genetic compatibility to viral host shifts, there are few well documented examples of bacterial pathogens adapting to novel hosts (Viana *et al.* 2015).

In recent decades, molecular analyses have revealed host shifting by bacterial pathogens may be occurring more frequently than previously thought (Guinane *et al.* 2010; Kraaijeveld *et al.* 2011; Lowder *et al.* 2009; Weinert *et al.* 2012). For example, phylogenetic analyses suggest *Wolbachia* bacteria independently colonized multiple species of arthropods via horizontal transmission (Kraaijeveld *et al.* 2011; Werren *et al.* 2008), a finding reaffirmed by empirical data *(Le Clec'h et al. 2012)*. *Staphylococcus aureus* similarly exhibits a diverse host range including poultry, ruminants, and other mammals, likely the result of host shifting from humans (Lowder *et al.* 2009; Weinert *et al.* 2012). However, identifying the specific factors contributing to bacterial host shifts remains challenging, as unlike viruses, bacteria must also be capable of extracting essential metabolic substrates, nutrients, and enzymatic cofactors such as iron from

their host (van Baarlen *et al.* 2007). Furthermore, depending on whether a bacterial pathogen exhibits extracellular and/or intracellular life cycle stages, it may also face a different suite of host immune defenses (van Baarlen *et al.* 2007). For instance, the colonization of the squid *Euprymna scolopes* by its bioluminescent symbiont *Vibrio fischeri* depends on the bacterium's ability to resist host immune defenses and environmental fluctuations through biofilm formation (Hussa *et al.* 2008). In other cases, such as is seen with *S. aureus*, a single mutation can underlie a cross-species host jump (Viana *et al.* 2015). In cases where the significance of accumulated mutations following a host shift is unclear, first examining the role of host exposure can not only help tease apart the relative roles of ecological and genetic factors, but also inform on any functional adaptations necessary for persistence, replication, and transmission among the novel host.

In recent decades, one of the most notable successful host shifts by bacterial pathogens occurred when *Mycoplasma gallisepticum* (*Mg*) emerged in house finches (*Haemorhous mexicanus*) in the mid 1990s. Comparative genomic analyses confirmed the host shift of *Mg* from chickens to house finches (Dhondt *et al.* 1998; Fischer *et al.* 1997; Ley *et al.* 1996) resulted from a single host shift event in eastern North America in the late 20th century (Delaney *et al.* 2012). The subsequent spread of *Mg* throughout North American house finches was uniquely well documented due to the externally visible conjunctivitis symptoms, quick identification of *Mg* as the causative agent, and active disease monitoring (Fischer *et al.* 1997; Ley *et al.* 1996). This host shift ultimately gave rise to a widespread epizootic that killed millions of house finches (Nolan *et al.* 1998). Since then, spillover infections have been documented in numerous other wild bird species (Dhondt *et al.* 2014; Farmer *et al.* 2005), heightening the need to understand

the relative importance of host exposure versus genetic factors in the original host shift into house finches.

Here, we investigated the role exposure in the host shift of *Mg* from poultry to house finches. To this end, we experimentally inoculated house finches with *Mg* strains sampled from either the original poultry host (Rlow) or the novel house finch host (epizootic outbreak HF1995 strain). Birds were then monitored for the establishment and progression of infection. If exposure was the key limiting factor in this host shift, then Rlow and HF1995 *Mg* should display similar abilities at establishing infection and causing clinical disease in the novel finch host. Conversely, a reduced or lack of infection by Rlow relative to HF1995 *Mg*, would indicate that genetic changes were also necessary for pathogen emergence in the novel finch host.

Materials and methods

House finch capture, housing, and experimental infection

We trapped male house finches at bird feeder sites in Alabama, USA, between August-September 2014 (as described in (Hill 2002). All birds used in the study were yearlings, having hatched in the spring of the calendar year in which they were collected. We collected birds from three sites in Auburn approximately 1.5 miles apart and two sites in Birmingham separated by 8 miles. Upon capture, a blood sample (~70ul) and choanal swab was collected from each bird. Blood plasma was used for a serum plate agglutination assay to test for anti-MG antibodies, indicating prior *Mg* exposure (Luttrell *et al.* 1996). Swabs were used for PCR amplification of *Mg* DNA to test for current infection (Roberts *et al.* 2001). Birds positive for either test were immediately released and not retained for the experiment. The remaining birds then underwent a 30-day quarantine period, during which they were treated for infection by *Trichomonas gallinae*

and *Coccidia spp*. Following quarantine, birds were randomly divided into treatment groups. Males in one treatment group were inoculated with an epizootic outbreak house finch *Mg* strain from 1995 (HF1995; passage 13) (N=15). Males in the second treatment group were inoculated with a poultry *Mg* strain (Rlow; passage 17) *Mg* (N=15). We inoculated birds by dropping 10 µl of the respective *Mg* culture into each eye, each containing approximately 1 x 10^4 to 1 x 10^6 color-changing units/ml of *Mg*. The HF1995 *Mg* isolate was cultured from a naturally infected house finch caught in Georgia, USA, during 1995. Dr. Naola Ferguson-Noel of the University of Georgia provided the Rlow *Mg* isolate. A third group of males from the quarantine group was assigned to a control treatment; we inoculated males in this group (N=11) by dropping 10 ul of sterile SP4 media into each eye. To prevent *Mg* transmission between treatments, we housed finches in separate rooms under identical conditions. Following inoculation, we monitored finches for the development of infection for 8 weeks (56 days). We also took a choanal (tracheal) swab sample on these days to test for the establishment of an *Mg* infection and pathogen load using quantitative PCR.

Quantification of clinical symptom severity

To document clinical symptoms, we photographed the right and left eyes of each bird, with the bird's eye parallel to the camera. We then quantified the area of the conjunctiva swelling in the photographs using the programs TpsUtil ver. 1.46 and TpsDig ver. 2.16 http://life.bio.sunysb.edu/morph/ (Rohlf 2010a; Rohlf 2010b). Each photograph was initially scaled (in mm) using an internal, known-length measurement for each bird, bill depth. The scaled picture files were then duplicated, with one file used for the placement of ten landmarks around the inner ring of the conjunctiva. The duplicate file was used to place twelve landmarks

around the outer area of the conjunctiva. Area measurements (mm^2) for the outer and inner rings of the conjunctiva were generated using TpsUtil. The area of the conjunctiva was then calculated as the area of the outer ring minus the area of the inner ring. To determine swelling severity, we subtracted the conjunctiva area at Day 0 (pre-inoculation) for a given individual from the area measured at a given sampling time point for that same individual. We estimated the background variation in our measurements by repeating this process with photographs of control birds, using twice the average background variation as the threshold for considering birds to display clinical conjunctiva swelling. The threshold value was subtracted from all measurements, with any values below the threshold being treated as having no, or zero, change in swelling.

MG presence and load

Choanal swabs were tested for the presence of MG via PCR (Roberts *et al.* 2001). Briefly, swabs were placed in 100 µl of sterile nuclease free water. Swabs were then placed at 100 °C for 10 minutes, at -20 °C for 10 minutes, and finally centrifuged at 13000 rpm for 5 minutes. We tested the supernatant of each sample in duplicate for MG presence using the forward primer 5' GCTTCCTTGCGGTTAGCAAC 3' and reverse primer 5' GAGCTAATCTGTAAAGTTGGTC 3'. PCR parameters were as follows: 94° C for 5 minutes, 35 cycles of 94° C for 30 seconds, 55° C for 30 seconds, and 72° C for 30 seconds, and a final 5 minute extension at 72° C (Roberts *et al.* 2001). In each assay, MG DNA extracted from pure culture served as a positive control.

We quantified tracheal MG loads using a TaqMan qPCR amplification of the single-copy MG gene, *mgc2*. To control for variation in the amount of starting material, we also amplified a

single copy house finch gene, *rag1* (Grodio *et al.* 2008). Before use, we cleaned up extracted swab samples using a Qiagen QIAquick PCR purification kit. All reactions were run on an ABI Prism 7500 (Applied Biosystems). We made a standard curve of pooled genomic DNA to estimate the relative amount of MG between individuals. We then divided the number of *mgc2* genes by one half the number of *rag1* genes to approximate the ratio of MG cells (haploid) to host cells (diploid).

Statistical analyses

All statistical analyses were conducted in R (R Core Team, 2012). We used lme4 (Bates, Maechler & Bolker, 2012) to perform a mixed effects logistic regression analysis of the relationship between infection status (infected or not with *Mg*) and treatment (Rlow *vs*. HF1995). As fixed effects, we entered treatment and time (dpi) and their interaction into the model, and intercepts for individuals was entered as a random effect. Differences in *Mg* persistence were tested using a general linear model with duration of *Mg* detection (using PCR) as the dependent variable and treatment as the explanatory factor. We tested for differences in the probability of developing clinical symptoms using a logistic regression with clinical symptoms development (yes/no) as the dependent variable and treatment (Rlow *vs.* HF1995) as the explanatory term. Differences in the severity of clinical symptoms were tested using a general linear model with maximum clinical symptoms reached as the dependent variable and treatment (Rlow *vs.* HF1995) as the explanatory term. We could not test for differences in clinical symptoms over time due to the small number of Rlow-inoculated birds that became symptomatic but show how these changed over the 56 days of the experiment in Fig 2. We tested for differences in the

maximum bacterial load reached using a general linear model with maximum *Mg* load as the dependent variable and treatment (Rlow *vs.* HF1995) as the explanatory term.

Results

All 15 birds inoculated with HF1995 became infected, while Rlow successfully established an infection in only 12 of 15 (80%) inoculated house finches. The probability of being infected with *Mg* significantly decreased over the course of the experiment for both treatments ($z=-4.5$, $p<0.0001$, estimate = -0.04 ± 0.009) (Fig. 1). In addition, Rlow-inoculated birds were significantly less likely to be infected than those inoculated with HF 1995 ($z = -4.9$, $p < 0.0001$, estimate = -1.6 ± 0.33) (Fig. 1). Furthermore, Rlow was able to persist in house finches for significantly shorter lengths of time than HF1995 (Rlow = 9.8 ± 1.8 days; HF1995 = 29.9 ± 4.1 days; $t = -3.9$, $p = 0.0006$). While 14 out of 15 finches (93%) inoculated with HF1995 displayed clinical symptoms, only 5 out of 15 (33%) Rlow-inoculated individuals became symptomatic (Fig 2). This difference was significant: Rlow-inoculated individuals exhibited a significantly lower probability of developing clinical symptoms than those inoculated with HF1995 ($z = -2.9$, $p = 0.004$, estimate = -3.3 ± 1.17). In addition, at their most severe, the clinical symptoms of Rlow-inoculated birds were significantly milder than that of HF1995 finches ($t = -7.0$, $p < 0.0001$, estimate = -3.13 ± 0.45) (Fig 2). Finally, birds inoculated with HF1995 developed higher bacterial loads than those inoculated with Rlow ($t = -5.9$, $p < 0.0001$, estimate = -2.23 ± 0.38) (Fig 3).

Discussion

Our results demonstrate that exposure alone was insufficient to allow for *Mg* to shift hosts and that host shifting by *Mg* likely involved adaptation to the novel host following exposure and colonization. The poultry *Mg* strain, Rlow, was able to establish an infection in 80% of inoculated house finches. However, these infections persisted for significantly shorter lengths of time than did those produced by the house finch epizootic strain, HF1995. Furthermore, Rlow achieved significantly lower bacterial loads at the site of infection and was less likely to give rise to clinical symptoms in the house finch host. When it did, symptoms remained significantly milder than those caused by HF1995. Given Rlow did not display an ability to infect or cause disease at comparable levels to HF1995, this indicates exposure was not the key limiting factor in this host shift.

Despite the need for adaptation to the house finch host, Rlow was able to successfully colonize and, as evidenced by an increase in bacterial load at day 7, establish an infection in the finch host. This processes of colonization and establishment of an infection requires the cytoadherence of *Mg* to the host epithelium and is under the control of several proteins that interact and bind the host extracellular matrix (Indikova *et al.* 2013; Jenkins *et al.* 2007). For instance, the attenuated *Mg* strain, Rhigh, exhibits low levels of cytadherence and minimal if any pathology in poultry. Analyses suggest this attenuation is related to the loss of expression of GapA and CmrA, both of which are involved in cytadherence, as well as HatA, part of an ABC transport system (Papazisi 2003; Papazisi *et al.* 2002). In other instances, in vitro siRNA knockdown of adhesin molecules, such as pMGA 1.2, has been shown to inhibit *Mg*-induced cell cycle disruption and damage to the tracheal epithelium. However, siRNA knockdown of poultry host cell receptor, ApoA1, also produces a similar effect (Hu *et al.* 2016). Although whole

genome analyses show that, relative to Rlow, house finch *Mg* strains possess mutations in genes that function in adherence to the host epithelia (Delaney *et al.* 2012; Tulman *et al.* 2012), given Rlow successfully infected house finches this suggests mutations in these genes were unlikely to have been a prerequisite for *Mg* shifting hosts into house finches.

The reduced ability of Rlow to persist and replicate within house finches relative to HF1995 suggests Rlow was likely compromised in its ability to evade and/or manipulate the house finch immune system. If true, we would expect Rlow and HF1995 to differ at genes involved in immune escape/manipulation as these genes would be under strong selection once in the novel finch host. Whole genome surveys of multiple house finch strains sampled over the first 12 years of the epizootic suggest that this may indeed have been the case (Delaney *et al.* 2012; Tulman *et al.* 2012). These analyses show the variable surface lipoprotein (*vlha*) genes, which are involved in host immune defense evasion through antigenic variation, represent most of the variation between the coding regions of the poultry and house finch *Mg* genomes (Chambaud *et al.* 1999; Delaney *et al.* 2012; Markham *et al.* 1998; Tulman *et al.* 2012). Similarly, most of the variation among house finch *Mg* strains was also detected within the *vlha* loci (Tulman *et al.* 2012), suggesting genes involved in interactions with the house finch immune system may have been under selection in the novel host.

While the ability to evade the host immune system is crucial to persistence, the reduced ability of Rlow to cause disease symptoms in the house finch host suggests Rlow may also be compromised in its ability to modulate house finch immune responses. In poultry, virulent *Mg* strains such Rlow have also been shown to induce greater dis-regulation of host immune responses than attenuated strains (Indikova *et al.* 2013; Majumder *et al.* 2014; Mohammed *et al.* 2007). Rlow has been show to induce a greater degree of macrophage chemotaxis (Majumder *et*

al. 2016) and expression of pro-inflammatory chemokines and cytokines (Majumder *et al.* 2014; Mohammed *et al.* 2007), which contribute to the disease-associated pathology seen in *Mg*-infected poultry (Gaunson *et al.* 2000, 2006). Although the house finches used in this study had no prior exposure to *Mg*, they originated from eastern US populations demonstrated to have evolved *Mg* resistance (Bonneaud *et al.* 2011). Resistance was found to be associated with milder conjunctivitis and mediated by the ability to escape pathogen-induced immuno-suppression. This was further associated with the development of an enhanced innate and specific cell-mediated immune response (Bonneaud *et al.* 2011; Bonneaud *et al.* 2012). Taken together with our findings that Rlow-inoculated birds displayed both milder symptoms and faster pathogen clearance, this suggests adaptations for both immune evasion and manipulation were likely crucial for *Mg* persistence and transmission within the novel house finch host population.

The need for such adaptive genetic changes could provide an explanation for why *Mg* appears to be jumping into alternative host populations at relatively high frequencies, but not persisting within these novel host species (Dhondt *et al.* 2008; Farmer *et al.* 2005; Stallknecht *et al.* 1998). Infection of other host species is therefore thought to reflect spillover events from natural host reservoirs, such as from wild house finches into other passerines (Hartup *et al.* 2001). In support, a phylogenetic analysis of 107 *Mg* strains from poultry, house finches, and other songbirds revealed that all strains obtained from non-house finch songbirds clustered with house finch strains (Hochachka et al. 2013). Furthermore, while house finch *Mg* can infect of a diverse array of passerines, it is only pathogenic (i.e., causes conjunctivitis) in other members of the family Fringillidae, such as in purple finches (*Haemorhous purpureus*) and American goldfinches (*Carduelis carduelis*) (Dhondt *et al.* 2008; Farmer *et al.* 2005). Indeed, evidence for *Mg* exposure, either via positive PCR-based detection of *Mg* or positive antibody tests, was

found in 27 species of wild birds representing 15 families, but clinical disease symptoms were rare or completely absent in most species (Dhondt *et al.* 2014). Taken together, these studies suggest that transmission is occurring regularly between many avian taxa as a result of exposure, but exposure alone is insufficient to give rise to a successful host shift. This is consistent with our findings: our results indicate that poultry *Mg* is able to colonize mucosal surfaces in house finches, but not persist or cause pathogenesis in the novel finch host at levels comparable to house finch *Mg* strains.

This study shows exposure to the novel host, while a prerequisite for host shifting, was not the key limiting factor in the jump of *Mg* from poultry to house finches. Our results are consistent with a phylogenetic study that found a singular *Mg* strain collected from an asymptomatic house finch to be more closely related to poultry *Mg* strains than house finch *Mg* strains (Hochachka *et al.* 2013). Given the sampling of *Mg* strains from house finches is both sporadic and conducted random, finding an Mg strain closely related to poultry in house finches should be extremely unlikely if exposure was the key limiting factor in the host shift. This finding, along with the extensive number of house finch *Mg* strains obtained from asymptomatic individuals of other passerine species, suggest that spill-overs of chicken *Mg* into house finches, or of house finch *Mg* into other passerines, may be more frequent than anticipated. The unique outbreak of severe conjunctivitis in house finches, however, suggests the adaptive genetic changes required for a successful host shift may be extremely rare.

Acknowledgements

This study was approved by Auburn University's IACUC under PRN 2014-2517. It was funded by a Natural Environment Research Council (NERC) grant to CB (NE/M00256X/1) and

small research grants to MS from the Alabama Ornithological Society, American Ornithologists' Union, Birmingham Audubon Society, Society for Integrative and Comparative Biology, and Wilson Ornithological Society. Additionally, an Auburn University Cellular and Molecular Bisociences Peaks of Excellence Research Fellowship provided support for MS while conducting this study. We would like to thank Dr. Wendy R. Hood, Dr. Mark R. Liles, Anna M. Tucker, P. Sankey, R. Mills, and Hill lab undergraduate students for assistance with this project. We also thank Hill and Hood lab members for providing valuable feedback on this manuscript.

References

Bonneaud C, Balenger S, Russell AF*, et al.* (2011) Rapid evolution of disease resistance is accompanied by functional changes in gene expression in a wild bird. *Proc Natl Acad Sci U S A* **108**, 7866-7871.

Bonneaud C, Balenger SL, Zhang J, Edwards SV, Hill GE (2012) Innate immunity and the evolution of resistance to an emerging infectious disease in a wild bird. *Mol Ecol* **21**, 2628-2639.

Chambaud I, Wroblewski H, Blanchard A (1999) Interactions between mycoplasma lipoproteins and the host immune system. *Trends in Microbiology* **7**, 493-499.

Chen H, Smith GJ, Zhang SY*, et al.* (2005) Avian flu: H5N1 virus outbreak in migratory waterfowl. *Nature* **436**, 191-192.

Delaney NF, Balenger S, Bonneaud C*, et al.* (2012) Ultrafast evolution and loss of CRISPRs following a host shift in a novel wildlife pathogen, *Mycoplasma gallisepticum*. *PLoS Genet* **8**, e1002511.

Dhondt AA, DeCoste JC, Ley DH, Hochachka WM (2014) Diverse wild bird host range of *Mycoplasma gallisepticum* in eastern North America. *PLoS One* **9**, e103553.

Dhondt AA, Dhondt KV, McCleery BV (2008) Comparative infectiousness of three passerine bird species after experimental inoculation with *Mycoplasma gallisepticum*. *Avian Pathol* **37**, 635-640.

Dhondt AA, Tessaglia DL, Slothower RL (1998) Epidemic mycoplasmal conjunctivitis in house finches from Eastern North America. *J Wildl Dis* **34**, 265-280.

Engering A, Hogerwerf L, Slingenbergh J (2013) Pathogen-host-environment interplay and disease emergence. *Emerg Microbes Infect* **2**, e5.

Farmer KL, Hill GE, Roberts SR (2005) Susceptibility of wild songbirds to the house finch strain of Mycoplasma gallisepticum. *J Wildl Dis* **41**, 317-325.

Fischer JR, Stallknecht DE, Luttrell MP, Dhondt AA, Converse KA (1997) Mycoplasmal conjunctivitis in wild songbirds: the spread of a new contagious disease in a mobile host population. *Emerg Infect Dis* **3**, 69-72.

Gaunson JE, Philip CJ, Whithear KG, Browning GF (2000) Lymphocytic infiltration in the chicken trachea in response to *Mycoplasma gallisepticum* infection. *Microbiology-Uk* **146**, 1223-1229.

Gaunson JE, Philip CJ, Whithear KG, Browning GF (2006) The cellular immune response in the tracheal mucosa to Mycoplasma gallisepticum in vaccinated and unvaccinated chickens in the acute and chronic stages of disease. *Vaccine* **24**, 2627-2633.

Grodio JL, Dhondt KV, O'Connell PH, Schat KA (2008) Detection and quantification of *Mycoplasma gallisepticum* genome load in conjunctival samples of experimentally

infected house finches (*Carpodacus mexicanus*) using real-time polymerase chain reaction. *Avian Pathol* **37**, 385-391.

Guinane CM, Ben Zakour NL, Tormo-Mas MA*, et al.* (2010) Evolutionary genomics of *Staphylococcus aureus* reveals insights into the origin and molecular basis of ruminant host adaptation. *Genome Biol Evol* **2**, 454-466.

Hartup BK, Dhondt AA, Sydenstricker KV, Hochachka WM, Kollias GV (2001) Host range and dynamics of mycoplasmal conjunctivitis among birds in North America. *J Wildl Dis* **37**, 72-81.

Hill GE (2002) *A red bird in a brown bag : the function and evolution of colorful plumage in the House Finch* Oxford University Press, Oxford ; New York.

Hochachka WM, Dhondt AA, Dobson A*, et al.* (2013) Multiple host transfers, but only one successful lineage in a continent-spanning emergent pathogen. *Proc Biol Sci* **280**, 20131068.

Hu F, Zhao C, Bi D*, et al.* (2016) *Mycoplasma gallisepticum* (HS strain) surface lipoprotein pMGA interacts with host apolipoprotein A-I during infection in chicken. *Appl Microbiol Biotechnol* **100**, 1343-1354.

Hussa EA, Darnell CL, Visick KL (2008) RscS functions upstream of SypG to control the syp locus and biofilm formation in *Vibrio fischeri*. *J Bacteriol* **190**, 4576-4583.

Imai M, Kawaoka Y (2012) The role of receptor binding specificity in interspecies transmission of influenza viruses. *Curr Opin Virol* **2**, 160-167.

Imai M, Watanabe T, Hatta M*, et al.* (2012) Experimental adaptation of an influenza H5 HA confers respiratory droplet transmission to a reassortant H5 HA/H1N1 virus in ferrets. *Nature* **486**, 420-428.

Indikova I, Much P, Stipkovits L*, et al.* (2013) Role of the GapA and CrmA cytadhesins of *Mycoplasma gallisepticum* in promoting virulence and host colonization. *Infect Immun* **81**, 1618-1624.

Jenkins C, Geary SJ, Gladd M, Djordjevic SP (2007) The *Mycoplasma gallisepticum* OsmC-like protein MG1142 resides on the cell surface and binds heparin. *Microbiology* **153**, 1455-1463.

Kerr PJ (2012) Myxomatosis in Australia and Europe: A model for emerging infectious diseases. *Antiviral Res* **93**, 387-415.

Kerr PJ, Ghedin E, DePasse JV*, et al.* (2012) Evolutionary history and attenuation of myxoma virus on two continents. *PLoS Pathog* **8**, e1002950.

Kraaijeveld K, Franco P, de Knijff P, Stouthamer R, van Alphen JJ (2011) Clonal genetic variation in a *Wolbachia*-infected asexual wasp: horizontal transmission or historical sex? *Mol Ecol* **20**, 3644-3652.

Kramer LD, Bernard KA (2001) West Nile virus infection in birds and mammals. *Ann N Y Acad Sci* **951**, 84-93.

Lambrechts L (2010) Dissecting the genetic architecture of host-pathogen specificity. *PLoS Pathog* **6**, e1001019.

Le Clec'h W, Braquart-Varnier C, Raimond M*, et al.* (2012) High virulence of *Wolbachia* after host switching: when autophagy hurts. *PLoS Pathog* **8**, e1002844.

Leroy EM, Kumulungui B, Pourrut X*, et al.* (2005) Fruit bats as reservoirs of Ebola virus. *Nature* **438**, 575-576.

Ley DH, Berkhoff JE, McLaren JM (1996) *Mycoplasma gallisepticum* isolated from house finches (*Carpodacus mexicanus*) with conjunctivitis. *Avian Dis* **40**, 480-483.

Liu J, Xiao H, Lei F*, et al.* (2005) Highly pathogenic H5N1 influenza virus infection in migratory birds. *Science* **309**, 1206.

Lowder BV, Guinane CM, Ben Zakour NL*, et al.* (2009) Recent human-to-poultry host jump, adaptation, and pandemic spread of *Staphylococcus aureus*. *Proc Natl Acad Sci U S A* **106**, 19545-19550.

Luttrell MP, Fischer JR, Stallknecht DE, Kleven SH (1996) Field investigation of *Mycoplasma gallisepticum* infections in house finches (*Carpodacus mexicanus*) from Maryland and Georgia. *Avian Dis* **40**, 335-341.

Maines TR, Chen LM, Matsuoka Y*, et al.* (2006) Lack of transmission of H5N1 avian-human reassortant influenza viruses in a ferret model. *Proc Natl Acad Sci U S A* **103**, 12121-12126.

Majumder S, Zappulla F, Silbart LK (2014) Mycoplasma gallisepticum lipid associated membrane proteins up-regulate inflammatory genes in chicken tracheal epithelial cells via TLR-2 ligation through an NF-kappaB dependent pathway. *PLoS One* **9**, e112796.

Majumder S, Zappulla F, Silbart LK (2016) Interaction of *Mycoplasma gallisepticum* with chicken tracheal and epithelial cells contributes to macrophage chemotaxis and activation. *Infection and Immunity* **84**, 266-274.

Markham PF, Glew MD, Browning GF, Whithear KG, Walker ID (1998) Expression of two members of the pMGA gene family of *Mycoplasma gallisepticum* oscillates and is influenced by pMGA-specific antibodies. *Infect Immun* **66**, 2845-2853.

Mohammed J, Frasca S, Cecchini K*, et al.* (2007) Chemokine and cytokine gene expression profiles in chickens inoculated with *Mycoplasma gallisepticum* strains R-low or GT5. *Vaccine* **25**, 8611-8621.

Nolan PM, Hill GE, Stoehr AM (1998) Sex, size, and plumage redness predict house finch survival in an epidemic. *Proceedings of the Royal Society B-Biological Sciences* **265**, 961-965.

Papazisi L (2003) The complete genome sequence of the avian pathogen *Mycoplasma gallisepticum* strain Rlow. *Microbiology* **149**, 2307-2316.

Papazisi L, Frasca S, Gladd M*, et al.* (2002) GapA and CrmA coexpression Is essential for *Mycoplasma gallisepticum* cytadherence and virulence. *Infection and Immunity* **70**, 6839-6845.

Park M, Loverdo C, Schreiber SJ, Lloyd-Smith JO (2013) Multiple scales of selection influence the evolutionary emergence of novel pathogens. *Philos Trans R Soc Lond B Biol Sci* **368**, 20120333.

Parrish CR, Holmes EC, Morens DM*, et al.* (2008) Cross-species virus transmission and the emergence of new epidemic diseases. *Microbiol Mol Biol Rev* **72**, 457-470.

Roberts SR, Nolan PM, Hill GE (2001) Characterization of *Mycoplasma gallisepticum* infection in captive house finches (*Carpodacus mexicanus*) in 1998. *Avian Dis* **45**, 70-75.

Rohlf FJ (2010a) TpsDig 2.

Rohlf FJ (2010b) TpsUtil: File Utility Program.

Stallknecht DE, Luttrell MP, Fischer JR, Kleven SH (1998) Potential for transmission of the finch strain of *Mycoplasma gallisepticum* between house finches and chickens. *Avian Dis* **42**, 352-358.

Taubenberger JK, Kash JC (2010) Influenza virus evolution, host adaptation, and pandemic formation. *Cell Host Microbe* **7**, 440-451.

Tulman ER, Liao X, Szczepanek SM*, et al.* (2012) Extensive variation in surface lipoprotein gene content and genomic changes associated with virulence during evolution of a novel North American house finch epizootic strain of *Mycoplasma gallisepticum*. *Microbiology* **158**, 2073-2088.

van Baarlen P, van Belkum A, Summerbell RC, Crous PW, Thomma BP (2007) Molecular mechanisms of pathogenicity: how do pathogenic microorganisms develop cross-kingdom host jumps? *FEMS Microbiol Rev* **31**, 239-277.

Viana D, Comos M, McAdam PR*, et al.* (2015) A single natural nucleotide mutation alters bacterial pathogen host tropism. *Nat Genet* **47**, 361-366.

Weinert LA, Welch JJ, Suchard MA*, et al.* (2012) Molecular dating of human-to-bovid host jumps by *Staphylococcus aureus* reveals an association with the spread of domestication. *Biol Lett* **8**, 829-832.

Werren JH, Baldo L, Clark ME (2008) Wolbachia: master manipulators of invertebrate biology. *Nat Rev Microbiol* **6**, 741-751.

Woolhouse ME, Haydon DT, Antia R (2005) Emerging pathogens: the epidemiology and evolution of species jumps. *Trends Ecol Evol* **20**, 238-244.

Yen HL, Lipatov AS, Ilyushina NA*, et al.* (2007) Inefficient transmission of H5N1 influenza viruses in a ferret contact model. *J Virol* **81**, 6890-6898.

Figures

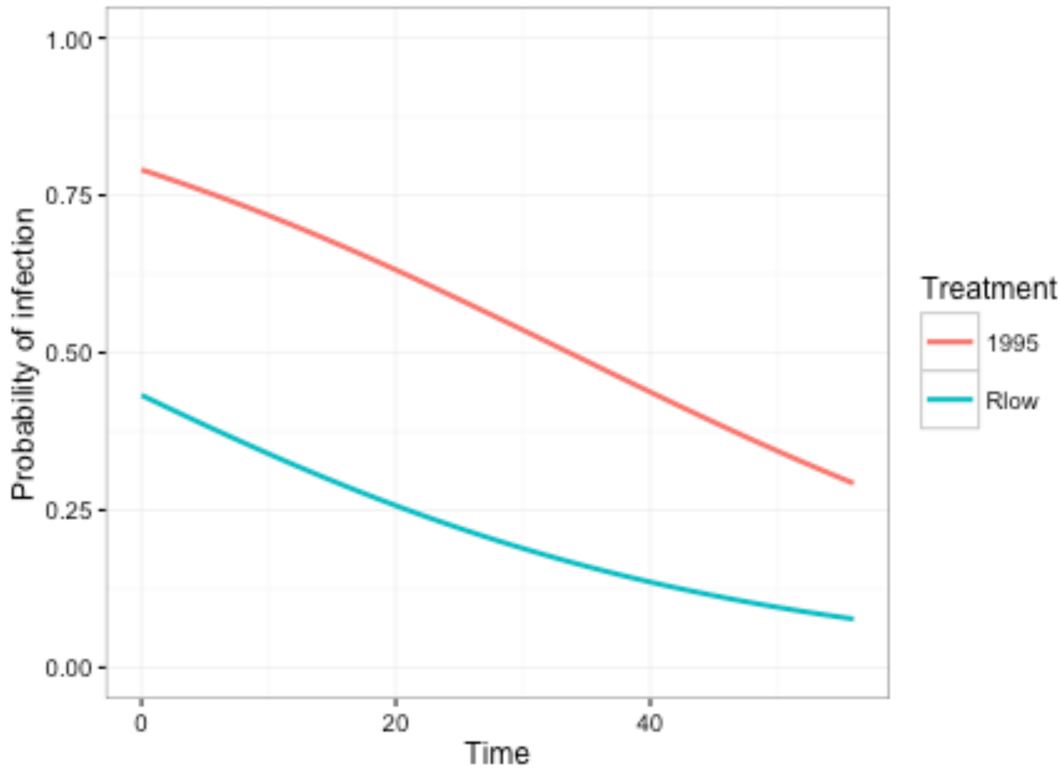

Figure 1. Probability of infection following inoculation with either a poultry strain of *Mg* (Rlow) or house finch epizootic-outbreak isolate (HF1995). We show the predicted values of the model (means ± SE).

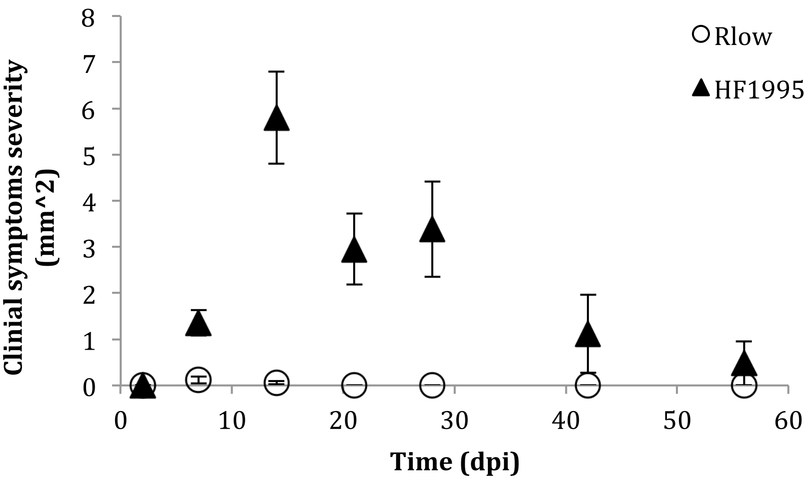

Figure 2. Development of clinical symptom over the course of the experiment (56 days post-infection (dpi)) in house finches inoculated with either a poultry strain of *Mg* (Rlow) or a house finch epizootic-outbreak isolate (HF1995). We show the means and standard errors.

(a)

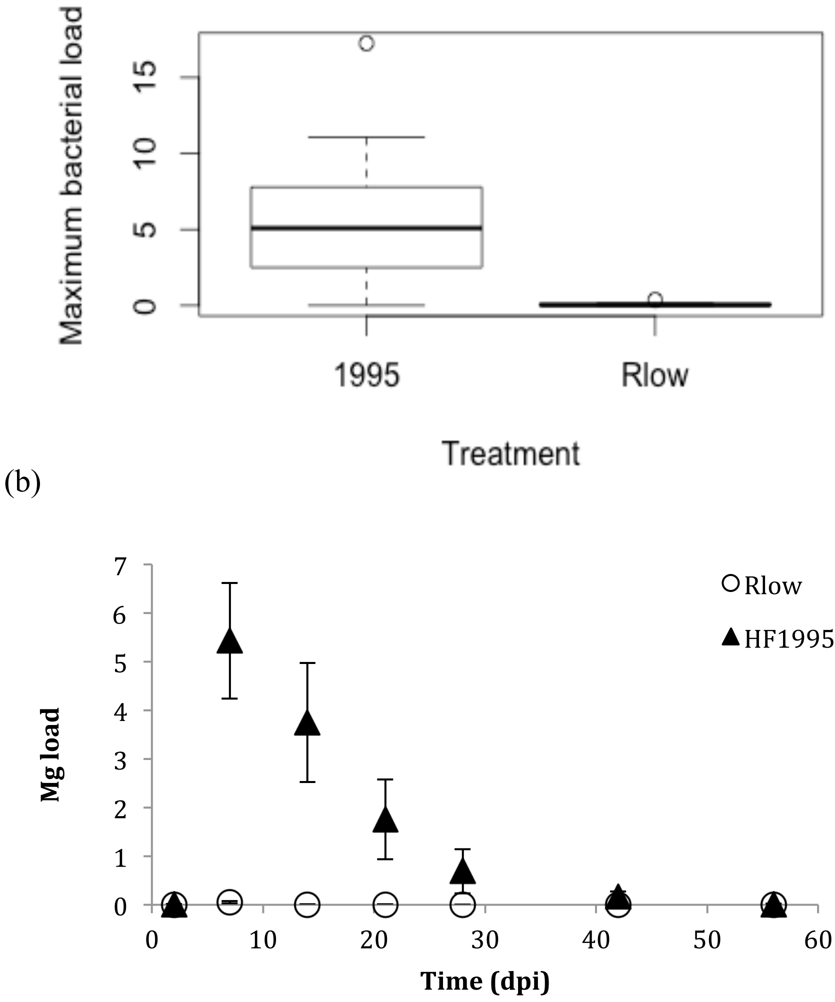

Figure 3. Bacterial load in the respiratory tract of house finches inoculated with a poultry strain of *Mg* (Rlow) or house finch epizootic-outbreak isolate (HF1995). We show raw values of *Mg* load expressed as a ratio of host cell number. (a) Maximum *Mg* load in each treatment group (poultry Rlow and house finch 1995); we show the median, 25th and 75th percentiles. (b) Changes in *Mg* load over the course of the experiment (56 days post-infection (dpi)); we show means and standard errors.

CHAPTER 4

Reduced ability of an emerging pathogen to infect the original donor host following a shift into a novel host

Abstract

Increasing emerging infectious disease outbreaks in recent decades make understanding factors influencing pathogen adaptation to novel host environments more crucial than ever. In particular, when both the original donor host and novel host are known, the capacity of a pathogen to re-infect a donor host can be utilized to assess whether adaptation to a novel host negatively influences a pathogen's host range. In this study, we examine the ability of *Mycoplasma gallistepticum* (*Mg*) to re-infect chickens (*Gallus gallus*) following a host shift into wild North American house finches (*Haemorhous mexicanus*). To test whether adaptation to house finches altered the ability of *Mg* to be transmitted back to the original poultry host, we experimentally inoculated chickens with an early epizootic strain of house finch *Mg* (HF 1994) or a virulent strain of poultry *Mg* (Rlow). HF1994 became established and persisted in 3 of 22 individuals for at least 14 days post-inoculation. However, HF1994 only caused minimal symptoms in one individual. In contrast, Rlow successfully became established in half of the infected individuals. While this reaffirms that house finch *Mg* is capable of re-infecting chickens, it also suggests adaptation to house finches negatively impacted *Mg's* ability to infect poultry. Furthermore, only 64% of Rlow-infected birds exhibited symptoms and only one individual

exhibited mass loss, suggesting some chickens may have been able to clear or suppress the pathogen to undetectable levels due to poultry *Mg* being poorly adapted to a conjunctiva transmission route. Taken together, these findings highlight that while *Mg* can be transmitted from house finches back to poultry, the poor adaptation of finch *Mg* to poultry will make such transmission rare.

Introduction

Adaptive evolution is the hallmark of natural selection; to thrive, a population must often evolve a suite of traits that enable organisms to better deal with their environment. For pathogenic microorganisms, a unique challenge is presented when adapting to the environment of a given host species. Not only must the pathogen adapt to the particular physiology of the host in order to extract the host's resources, but mechanisms for transmission and evasion of host immune defenses must also be developed and maintained (Antonovics *et al.* 2013). Consequently, when faced with a novel host environment, pathogens may undergo rapid selection for adaptations that influence virulence, transmission potential, and survivability (Schrag & Wiener 1995). Often, when a novel pathogen emerges, the focus is placed on elucidating factors that allowed the pathogen to overcome host barriers to infection and transmission (Lambrechts 2010; Lloyd-Smith *et al.* 2009; Rigaud *et al.* 2010; Woolhouse *et al.* 2005). Less well understood, however, is how the process of adaptation to one host environment may influence the capacity of a pathogen to infect other host species (Lloyd-Smith *et al.* 2009; Rigaud *et al.* 2010).

Disease emergence events can facilitate an understanding of how selective pressures imposed by a novel host environment can influence pathogen evolution and subsequently affect

adaptation to another host species. When disease emergence is the result of a pathogen host shift, examination of pathogen isolates from the novel and original donor host species can reveal information about whether a pathogen is able to re-infect its original host after adapting to the novel one. For instance, *Mycobacterium bovis*, the causative agent of tuberculosis in cattle, underwent a host shift from cattle to badgers (*Meles meles*) roughly a century ago and has since become well adapted to the novel badger host (Palmer 2007). Despite this adaptation to badgers, disease models and genetic evidence support ongoing transmission of *M. bovis* back to the original cattle host (Biek *et al.* 2012; Donnelly *et al.* 2006), presumably facilitated by badgers exploiting farm resources (Drewe *et al.* 2013; Garnett *et al.* 2002). This illustrates that despite adaptation to a distantly related host, a pathogen may still maintain the ability to infect its original host. Although transmission may occur, a given pathogen will not necessarily have equal ability to infect and persist in different host environments. For instance, in a large-scale cross-infection study that involved inoculating 48 species of Drosophilidae with *Drosophila* C Virus (DCV), virulence of DCV in the novel hosts was linked to both pathogen load and host phylogeny. What underlies the differences in DCV's ability to infect and cause disease in these host species remains unclear (Longdon *et al.* 2015). However, for some pathogens only a few key mutations are necessary for adaptation to a novel host environment. For instance, only a single nucleotide mutation is necessary for the human strain of *Staphylococcus aureus* to adapt to a novel rabbit host (Viana *et al.* 2015). Similarly, experimental adaptations of the pandemic 2009 H1N1 strain of highly pathogenic avian influenza (HPAI) to mouse cells (Ilyushina *et al.* 2010) and H5N1 to guinea pigs (Gao *et al.* 2009) involved amino acid substitutions in only two proteins: the hemagglutinin, which is involved in host receptor binding, and the PB2 subunit of the viral RNA polymerase (Gao *et al.* 2009; Ilyushina *et al.* 2010). Thus, in the face of strong

selection, those pathogens that face low mutational barriers to host shifting may be able to rapidly adapt to a novel host and cause potentially devastating disease outbreaks (Allison *et al.* 2012).

Here, we examine how shifting hosts from a gallinaceous bird (Galliformes) to a songbird (Passeriformes) altered the ability of the bacterium *Mycoplasma gallisepticum* (*Mg)* to infect its original poultry host. In 1994, *Mg* underwent a host shift into wild eastern North American house finches (*Haemorhous mexicanus*) (Fischer *et al.* 1997; Ley *et al.* 1996). Subsequent whole genome analyses of *Mg* isolates from both poultry and house finches indicated this epizootic was the result of a single host shift event accompanied by large-scale genomic changes (Delaney *et al.* 2012; Tulman *et al.* 2012), suggesting *Mg* underwent extensive adaptive evolution in the process of shifting hosts. Consistent with this idea, the virulent poultry *Mg* strain, Rlow, was recently shown to have the capacity to infect house finches, but caused minimal to no clinical disease symptoms (i.e., conjunctivitis) in infected individuals (Staley *et al. In prep*). These observations indicate that genetic adaptation was necessary for *Mg* to thrive within the novel house finch environment and subsequently give rise to an epizootic (Staley *et al. In prep*). However, whether or not the adaptive changes required for proliferation in a songbird host altered house finch *Mg'*s ability to thrive in the original poultry host remains unclear.

To date, a single experimental challenge of poultry with a house finch-adapted *Mg* strain showed that chickens (*Gallus gallus*) and turkeys (*Meleagris gallopavo)* can become infected with house finch *Mg* via aerosol exposure. Yet, when compared to individuals infected with chicken *Mg,* infected individuals exhibited mild to no clinical disease symptoms or pathology, as well as lower antibody titers (O'Connor *et al.* 1999). Given that transmission routes and tissue localization of this pathogen differ between house finch and poultry *Mg* strains, an aerosol-based

challenge may not accurately represent the likelihood of successful transmission of *Mg* from house finches back into poultry. In poultry, acute infection with *Mg* is primarily characterized by respiratory disease and sinusitis (Kerr & Olsen 1967; Lamas da Silva & Adler 1969; Thomas *et al.* 1966), although it has also been found to invade and survive within chicken red blood cells (Vogl *et al.* 2008; Winner *et al.* 2000) and thus spread systemically (Much *et al.* 2002). This systemic spreading can lead to such symptoms as edema, arthritis, splenomegaly, and encephalitis (Kerr & Olsen 1967; Lamas da Silva & Adler 1969; Thomas *et al.* 1966). *Mg* also localizes to tissues such as the conjunctiva, but associated symptoms (i.e., conjunctivitis) are observed relatively infrequently (Gharaibeh & Hailat 2011; Much *et al.* 2002; Nunoya *et al.* 1995). Despite house finch *Mg* also localizing to the respiratory tract, the main disease symptom displayed by *Mg*-infected house finches is conjunctivitis (Dhondt *et al.* 2005; Ley *et al.* 1996). The house finch *Mg* lineage is thought to be adapted for transmission through direct contact with an infected individual or indirectly through deposition of conjunctiva secretions on fomites, which has been shown to increase with increased conjunctiva swelling, independent of *Mg* load (Adelman *et al.* 2013; Dhondt *et al.* 2007). In support of this supposed route of transmission, when naturally infected house finches were housed in the same room as chickens but not allowed direct contact, no evidence for *Mg* transmission was found. In contrast, after prolonged direct contact (>10 weeks) with infected house finches, 80% of chickens seroconverted and infection with *Mg* was detected by PCR or culture in 20-30% of chickens, indicating transmission back to poultry from house finches (Stallknecht *et al.* 1998). Taken together, these studies suggest house finch *Mg* is likely best adapted for direct transmission via conjunctiva secretions, rather than aerosol-based transmission, unlike in the original host. Experimentally challenging chickens via

an eye-drop inoculation, would therefore provide a more accurate assessment of the potential for house finch *Mg* to re-infect chickens.

To evaluate the capacity of house finch *Mg* to re-infect the original poultry host via direct exposure to the conjunctiva, we experimentally infected chickens via eye drop inoculation with either a virulent poultry *Mg* isolate (Rlow) or an early epizootic house finch *Mg* isolate (HF1994) from Virginia, United States. If house finch *Mg* retained the ability to infect poultry following the host shift, we would expect similar infection probabilities for Rlow and HF1994. Alternatively, if the genetic changes in HF1994 relative to Rlow compromised the ability of HF1994 to infect poultry, we would predict reduced or no infection success with HF1994 compared to Rlow.

Materials and methods

Chickens, housing, and experimental infection

6-week-old specific pathogen free male white leghorn chickens were purchased through Charles River (www.criver.com) and housed in BSL-2 level containment at the Auburn University Veterinary Research Building at the Center for Veterinary Medicine. Chickens were fed *ad lib* using Charles River chicken feed to minimize effects due to diet change. Upon arrival, the chickens were given 72 hours to acclimate to the novel environment, during which time no procedures were conducted. Following the acclimation period, samples were taken to verify no prior exposure to *Mg*; blood plasma was used in a serum plate agglutination assay to test for anti-*Mg* antibodies, indicating prior *Mg* exposure, and choanal swabs were used for PCR amplification of *Mg* DNA to test for current infection (Roberts *et al.* 2001). We subsequently inoculated chickens with the known-virulent live poultry *Mg* strain, Rlow (N=22), provided by

Dr. Naola Ferguson-Noel of the University of Georgia, or an early epizootic house finch *Mg* strain from Virginia, HF1994 (N=22). We inoculated birds by dropping 10 µl of the respective *Mg* culture into each eye, each containing approximately 1 x 10^4 to 1 x 10^6 color-changing units/ml of *Mg*. Additionally, we inoculated a group of control birds (N=11) by dropping 10 ul of sterile SP4 media into each eye. Due to facility constraints, the experiment was conducted in three consecutive stages, with each stage representing a distinct treatment group. To minimize variation across treatments, all chickens were kept under identical conditions at this facility. Following inoculation, we monitored chickens for the development of infection for 14 days. Mass measurements were taken at 0, 2, 7, 11 and 14 days post-inoculation (dpi). At this time, we also noted whether individuals displayed clinical symptoms of disease such as conjunctival swelling, labored breathing, nasal discharge, sneezing, and edema. Blood samples collected at days 2, 7, and 14 as well as conjunctival and tracheal tissues collected at 14 dpi were used to test for infection persistence and systemic spreading. Animals were euthanized on day 14 dpi and conjunctiva and tracheal tissues were collected and preserved in RNAlater®.

Mg presence

Tissues collected from the trachea and conjunctiva after euthanasia and choanal swabs collected at 14 dpi were tested for the presence of *Mg* via PCR (Roberts *et al.* 2001). Briefly, DNA was extracted samples using a Qiagen DNeasy Blood and Tissue kit (Catolog No. 69506; https://www.qiagen.com/us/). We tested each sample in duplicate for *Mg* presence using the forward primer 5' GCTTCCTTGCGGTTAGCAAC 3' and reverse primer 5' GAGCTAATCTGTAAAGTTGGTC 3'. PCR parameters were as follows: 94° C for 5 minutes, 35 cycles of 94° C for 30 seconds, 55° C for 30 seconds, and 72° C for 30 seconds, and a final 5

minute extension at 72° C (Roberts *et al.* 2001). In each assay, *Mg* DNA extracted from pure culture served as a positive control.

Statistical analyses

All statistical analyses were conducted in R (R Core Team, 2012). We tested for differences in the probability of being infected with *Mg* using a logistic regression with presence of *Mg* (yes/no) as the dependent variable and treatment (Rlow *vs.* HF1994) as the explanatory term. Presence of Mg was determined as a positive PCR from choanal swabs, trachea or conjunctivae tissues at 14 dpi. We tested for differences in the probability of developing clinical symptoms using a logistic regression with clinical symptoms development (yes/no) as the dependent variable and treatment (Rlow *vs.* HF1994) as the explanatory term. We tested for differences in body mass over the course of the experiment using a ANCOVA with mass change at days 2, 7, 11 and 14 relative to mass at day 0 as the dependent variable and time, treatment (Rlow, HF1994 or control) and initial body mass (at day 0) as the explanatory terms. We used a similar model to test for differences in body mass change over time between Rlow and HF1994-infected chicken using an ANCOVA with mass change at days 2, 7, 11 and 14 relative to mass at day 0 as the dependent variable and time, treatment (Rlow *vs.* HF1994) and initial body mass (at day 0) as the explanatory terms.

Results

Chicken *Mg*, Rlow, successfully established an infection in 50% (11/22) of inoculated chickens, whereas the epizootic house finch *Mg* strain, HF1994, became established in 14% (3/22) of inoculated individuals. This difference was significant; Rlow-inoculated birds were

significantly more likely to be infected at 14 dpi than those inoculated with HF1994 ($z = -2.5$, $p = 0.014$, estimate = -1.9 ± 0.75) (Fig. 1). The difference in symptoms between Rlow and HF1994-infected chickens was also significant. Rlow-inoculated individuals exhibited a significantly higher probability of developing clinical disease than those inoculated with HF1995 ($z = -3.2$, $p = 0.001$, estimate = -3.6 ± 1.12). 64% (14/22) of chickens inoculated with Rlow displayed clinical symptoms, while only 5% (1/22) HF1994-inoculated individuals became symptomatic (Fig 2).

Qualitatively, clinical disease symptoms displayed by Rlow-inoculated individuals were highly variable and included pale combs, moderately labored breathing, sneezing, production of large amounts of nasopharyngeal mucus, and periocular exudate, erythema, and swelling. Additionally, one individual displayed pedal edema at 14 dpi. As no control individuals and only one individual inoculated with HF 1994 became symptomatic (as indicated by repeated sneezing), differences in the onset or severity of clinical symptoms between treatments could not be tested. Considering all individuals together, however, we found that the onset of clinical symptoms was variable. Four individuals began to display clinical symptoms at 7 dpi, and the remaining symptomatic individuals displayed symptoms at either 11 or 14 dpi. In contrast, the only symptomatic HF1994-infected bird showed clinical symptoms at 7 dpi, and no control individuals showed any clinical symptoms.

As expected due to age at the initiation of the experiment, all chickens gained body mass over the course of the experiment ($F = 2863.6$, $p < 0.0001$; Fig 3). Body mass changes were also significantly affected by treatment ($F = 18.2$, $p < 0.0001$) and initial body mass ($F = 18.3$, $p < 0.0001$), with control chickens gaining less mass over time than *Mg*-infected ones; the only exception was one Rlow-inoculated individual that exhibited mass loss between 7 and 14 dpi.

There was, however, no significant difference in mass gain between Rlow and HF1994-inoculated chickens (treatment: $F = 2.3$, $p = 0.13$).

Discussion

Previous studies have demonstrated that *Mg* underwent rapid and large-scale evolutionary changes in the host shift from poultry to house finches (Delaney *et al.* 2012; Tulman *et al.* 2012). Here, we show that the adaptive changes enabling *Mg* to become a successful pathogen of house finches consequently caused it to become a less effective pathogen of chickens. Indeed, while house finch *Mg* did infect a few individual chickens, it had both a lower probability of infection than poultry *Mg* and caused clinical symptoms in only one individual. Taken together, these results suggest the adaptations that facilitate the infection of a novel host species may come at a cost to a pathogen's ability to re-infect the original host species.

The process of host shifting may involve pathogen adaptations related to any of the complex series of steps involved in the infection process, beginning with adherence to host tissues and later obtaining resources from the host, evading host immune defenses, and being transmitted to the next host (Antonovics *et al.* 2013). For many pathogens, molecules involved in host attachment are crucial to determining their host range. For instance, host shifting of canine parvovirus between dogs and raccoons involves a few key amino acid changes in the capsid protein. Consequently, isolates from raccoons that do not possess these specific amino acid residues are unable to bind and infect canine host cells (Allison *et al.* 2012). However, in other cases other adaptations such as those for resisting host immune defenses may be more pertinent. For example, the colonization of the squid *Euprymna scolopes* by its bioluminescent symbiont *Vibrio fischeri* is dependent on biofilm formation, which confers enhanced resistance to host

immune defenses and environmental fluctuations (Hussa *et al.* 2008; Mandel *et al.* 2009). Which adaptations are necessary to infect and persist within a novel will depend the unique suite of challenges imposed by the novel environment.

In the case of *Mg,* whole genome analyses indicate adaptation to house finches was predominantly associated with mutations in genes that function in adherence to the host epithelia and evasion of the house finch immune system (Delaney *et al.* 2012; Szczepanek *et al.* 2010; Tulman *et al.* 2012). Host attachment is the critical first step in the infection process (Indikova *et al.* 2013; Jenkins *et al.* 2007) and interference with this step can have negative repercussions for *Mg's* ability to infect and cause disease in a given host. The critical nature of this step is illustrated by how, *in vitro*, *Mg*-induced cell cycle disruption and tracheal cilia damage can be inhibited by siRNA knockdown of pMGA 1.2 expression, an adhesin molecule of *Mg,* or its chicken host cell receptor, ApoA1 (Hu *et al.* 2016). However, a few HF1994-inoculated individuals were PCR-positive for *Mg* at 14 dpi and HF1994 was detected in the trachea of one individual. This suggests that rather than resulting in an altered ability to attach to the epithelium of the original host (i.e., chickens), adaptation to the novel house finch host may have resulted in a trade-off in the ability of *Mg* to manipulate and evade poultry immune defenses. Numerous studies of attenuated *Mg* strains have highlighted the importance of the ability of *Mg* to induce dis-regulation of host immune responses to the infection process (Indikova *et al.* 2013; Majumder *et al.* 2014, 2016; Mohammed *et al.* 2007). Compared to attenuated strains, Rlow induces greater up-regulation of pro-inflammatory chemokines and cytokines (Majumder *et al.* 2014; Mohammed *et al.* 2007) and media from tracheal epithelial cells exposed to Rlow induces a greater degree of macrophage chemotaxis (Majumder *et al.* 2016). Given this process underlies much of the disease pathology associated with *Mg* infections in poultry (Gaunson *et al.* 2000,

2006b), *Mg* may have faced strong selection to adapt to the novel house finch immune system at the cost of being able to evade and manipulate the immune defenses of the donor poultry host.

In addition to adaptations related to immune evasion and manipulation, a likely key adaptation that facilitated the colonization of house finches by *Mg* was the ability to efficiently infect the host upon exposure to host conjunctiva tissues. Despite Rlow having a higher probability of infecting chickens than HF1994, only 50% of individuals were still infected at 14 dpi and only 64% displayed any associated clinical disease symptoms. This low infection rate illustrates the importance of transmission route to the infection process, as poultry *Mg* tends to spread among chickens via aerosolized droplets. A consequence of Rlow being poorly adapted for this transmission route, then, may be that poultry are better able to mount and regulate an immune response to *Mg,* thereby reducing disease-associated pathology. Indeed, eye drop inoculation of Rlow into 9-week-old chickens does not result in measurable pathology as assessed by air sac lesions, tracheal mucosal thickness, or tracheal lesions between 1-3 weeks post-inoculation. In contrast, chickens infected via intra-tracheal inoculation exhibited consistent pathology (Leigh *et al.* 2012). Similarly, turkeys exposed to *Mg* via eye drop inoculation exhibit less severe pathology than those given an aerosol exposure (Lin & Kleven 1982). Taken together, this suggests poultry *Mg* is less able to cause clinical disease and consequently be spread to new individuals when transmitted via the conjunctiva instead of respiratory droplets.

Another hallmark of *Mg*-associated pathology in both house finches and poultry is often mass loss (Bonneaud *et al.* 2012; Lin & Kleven 1982), but with the exception of one Rlow-infected individual, neither HF1994- or Rlow-infected chickens exhibited mass loss relative to controls. These findings are in agreement with previous poultry studies that utilized eye drop inoculations (Ganapathy & Bradbury 2003; Leigh *et al.* 2012; Lin & Kleven 1982) and are

consistent with the idea that, in susceptible individuals, mass loss represents a cost of pathogenesis rather than a cost of disease resistance. For instance, when house finches from *Mg*-unexposed populations were experimentally challenged with *Mg*, those individuals that exhibited the greatest mass loss also had the highest pathogen loads (Bonneaud *et al.* 2012). Furthermore, this is consistent with both Rlow and HF1994 being compromised in their ability to infect chickens, either because of the reduced ability of Rlow to infect chickens via the conjunctiva and or the trade-off faced by *Mg* in its adaptation to house finches.

Although Rlow *Mg* arguably has a reduced capacity to infect poultry via the conjunctiva, factors related to the design of the experiment such as *Mg* dosage, sampling scheme, or host characteristics could also contribute to the observed patterns. When experimentally given low dosages (10^2 CFUs) of *Mg* via an intra-tracheal inoculation*,* some chickens exhibit detectable *Mg* levels only at early sampling time points (<14 dpi). In contrast, other chickens given this or a higher dosage (10^5 and 10^8 CFUs) exhibited increases in *Mg* from 3-5 dpi that subsequently declined beginning a 7 dpi, with *Mg* still detectable in many individuals at 35 dpi. Despite such a short duration of *Mg* detectability in some individuals, those individuals were still able to transmit Mg to unexposed chickens (Feberwee *et al.* 2005). Thus, even if *Mg* levels only transiently increased in those individuals negative for *Mg* by PCR at 14 dpi, transmission among poultry or between poultry and house finches could possibly still occur. Furthermore, although we found lower infection success with house finch *Mg* compared to prior experimental work (O'Connor *et al.* 1999), this difference illuminates the potential influence host characteristics such as breed of chicken (white leghorn versus broiler) and age at exposure (6 weeks versus 3 weeks) (O'Connor *et al.* 1999) may have on *Mg* transmission dynamics. Even within a breed, artificially selected poultry lines differ in their ability to generate protective immune responses

against *Mg* (Yagihashi *et al.* 1992). Additionally, chickens infected with *Mg* at less than 4 weeks of age reach higher pathogen loads, exhibit more severe clinical disease symptoms, and generated less effective protective immune responses than those infected initially at 4 or 6 weeks of age (Gaunson *et al.* 2006a). Therefore, host characteristics such as age may play an important role in determining the probability of a given individual becoming infected and/or symptomatic, which consequently may alter transmission dynamics among and between host species.

While our study reaffirms the ability of house finch *Mg* to infect poultry through direct eye drop exposure, such transmission is unlikely to occur and even more so, to give rise to sustained infection in the poultry population. To date, a single phylogenetic study found two *Mg* isolates whose haplotypes were suggestive of continued *Mg* transmission between poultry and house finches: one collected from an asymptomatic house finch found to be most closely related to poultry *Mg* isolates and one from poultry found to be most closely related to house finch *Mg* isolates (Hochachka *et al.* 2013). Continued sampling and analyses of *Mg* isolates from naturally infected species will be crucial for not only understanding the host range dynamics of *Mg,* but also identifying the key adaptive mutations that facilitated the initial colonization of house finches by *Mg* and the subsequent epizootic it created.

Acknowledgements

This study was approved by Auburn University's IACUC under PRN 2013-2324. It was funded by a Natural Environment Research Council (NERC) grant to CB (NE/M00256X/1) and an Auburn University HATCH Grant to GEH. Additionally, an Auburn University Cellular and Molecular Biosciences Peaks of Excellence Research Fellowship provided support for MS while conducting this study. We would like to thank Dr. Mark R. Liles, Rebecca Koch, Zhiyuan Ge,

David Morris, Chloe Josefson, Hilary Rizk, Evan Smallwood, Alan Webster, Adam Lucy, Ryan Weaver, Matthew Warren, Carla Stout, Malorie Hayes, Erika Keshishian, and numerous Hood lab undergraduate students for assistance with this project.

References

Adelman JS, Carter AW, Hopkins WA, Hawley DM (2013) Deposition of pathogenic *Mycoplasma gallisepticum* onto bird feeders: host pathology is more important than temperature-driven increases in food intake. *Biol Lett* **9**, 20130594.

Allison AB, Harbison CE, Pagan I*, et al.* (2012) Role of multiple hosts in the cross-species transmission and emergence of a pandemic parvovirus. *J Virol* **86**, 865-872.

Antonovics J, Boots M, Ebert D*, et al.* (2013) The origin of specificity by means of natural selection: evolved and nonhost resistance in host-pathogen interactions. *Evolution* **67**, 1-9.

Biek R, O'Hare A, Wright D*, et al.* (2012) Whole genome sequencing reveals local transmission patterns of *Mycobacterium bovis* in sympatric cattle and badger populations. *PLoS Pathog* **8**, e1003008.

Bonneaud C, Balenger SL, Hill GE, Russell AF (2012) Experimental evidence for distinct costs of pathogenesis and immunity against a natural pathogen in a wild bird. *Mol Ecol* **21**, 4787-4796.

Delaney NF, Balenger S, Bonneaud C*, et al.* (2012) Ultrafast evolution and loss of CRISPRs following a host shift in a novel wildlife pathogen, *Mycoplasma gallisepticum*. *PLoS Genet* **8**, e1002511.

Dhondt AA, Altizer S, Cooch EG*, et al.* (2005) Dynamics of a novel pathogen in an avian host: Mycoplasmal conjunctivitis in house finches. *Acta Trop* **94**, 77-93.

Dhondt AA, Dhondt KV, Hawley DM, Jennelle CS (2007) Experimental evidence for transmission of *Mycoplasma gallisepticum* in house finches by fomites. *Avian Pathol* **36**, 205-208.

Donnelly CA, Woodroffe R, Cox DR*, et al.* (2006) Positive and negative effects of widespread badger culling on tuberculosis in cattle. *Nature* **439**, 843-846.

Drewe JA, O'Connor HM, Weber N, McDonald RA, Delahay RJ (2013) Patterns of direct and indirect contact between cattle and badgers naturally infected with tuberculosis. *Epidemiol Infect* **141**, 1467-1475.

Feberwee A, Mekkes DR, Klinkenberg D*, et al.* (2005) An experimental model to quantify horizontal transmission of Mycoplasma gallisepticum. *Avian Pathol* **34**, 355-361.

Fischer JR, Stallknecht DE, Luttrell P, Dhondt AA, Converse KA (1997) Mycoplasmal conjunctivitis in wild songbirds: the spread of a new contagious disease in a mobile host population. *Emerg Infect Dis* **3**, 69-72.

Ganapathy K, Bradbury JM (2003) Effects of cyclosporin A on the immune responses and pathogenesis of a virulent strain of Mycoplasma gallisepticum in chickens. *Avian Pathology* **32**, 495-502.

Gao Y, Zhang Y, Shinya K*, et al.* (2009) Identification of amino acids in HA and PB2 critical for the transmission of H5N1 avian influenza viruses in a mammalian host. *PLoS Pathog* **5**, e1000709.

Garnett BT, Delahay RJ, Roper TJ (2002) Use of cattle farm resources by badgers (*Meles meles*) and risk of bovine tuberculosis (*Mycobacterium bovis*) transmission to cattle. *Proc Biol Sci* **269**, 1487-1491.

Gaunson JE, Philip CJ, Whithear KG, Browning GF (2000) Lymphocytic infiltration in the chicken trachea in response to *Mycoplasma gallisepticum* infection. *Microbiology-Uk* **146**, 1223-1229.

Gaunson JE, Philip CJ, Whithear KG, Browning GF (2006a) Age related differences in the immune response to vaccination and infection with Mycoplasma gallisepticum. *Vaccine* **24**, 1687-1692.

Gaunson JE, Philip CJ, Whithear KG, Browning GF (2006b) The cellular immune response in the tracheal mucosa to Mycoplasma gallisepticum in vaccinated and unvaccinated chickens in the acute and chronic stages of disease. *Vaccine* **24**, 2627-2633.

Gharaibeh S, Hailat A (2011) *Mycoplasma gallisepticum* experimental infection and tissue distribution in chickens, sparrows and pigeons. *Avian Pathol* **40**, 349-354.

Hochachka WM, Dhondt AA, Dobson A*, et al.* (2013) Multiple host transfers, but only one successful lineage in a continent-spanning emergent pathogen. *Proc Biol Sci* **280**, 20131068.

Hu F, Zhao C, Bi D*, et al.* (2016) *Mycoplasma gallisepticum* (HS strain) surface lipoprotein pMGA interacts with host apolipoprotein A-I during infection in chicken. *Appl Microbiol Biotechnol* **100**, 1343-1354.

Hussa EA, Darnell CL, Visick KL (2008) RscS functions upstream of SypG to control the syp locus and biofilm formation in *Vibrio fischeri*. *J Bacteriol* **190**, 4576-4583.

Ilyushina NA, Khalenkov AM, Seiler JP*, et al.* (2010) Adaptation of pandemic H1N1 influenza viruses in mice. *J Virol* **84**, 8607-8616.

Indikova I, Much P, Stipkovits L*, et al.* (2013) Role of the GapA and CrmA cytadhesins of *Mycoplasma gallisepticum* in promoting virulence and host colonization. *Infect Immun* **81**, 1618-1624.

Jenkins C, Geary SJ, Gladd M, Djordjevic SP (2007) The *Mycoplasma gallisepticum* OsmC-like protein MG1142 resides on the cell surface and binds heparin. *Microbiology* **153**, 1455-1463.

Kerr KM, Olsen NO (1967) Pathology in chickens experimentally inoculated with or contact-infected with *Mycoplasma gallisepticum*. *Avian Dis* **11**, 559-578.

Lamas da Silva JM, Adler HE (1969) Pathogenesis of arthritis induced in chickens by *Mycoplasma gallisepticum*. *Pathol Vet* **6**, 385-395.

Lambrechts L (2010) Dissecting the genetic architecture of host-pathogen specificity. *PLoS Pathog* **6**, e1001019.

Leigh SA, Branton SL, Evans JD, Collier SD (2012) Effect of infection route and concurrent infectious bronchitis virus vaccination on *Mycoplasma gallisepticum* disease pathology in an experimental model. *Avian Pathol* **41**, 497-503.

Ley DH, Berkhoff JE, McLaren JM (1996) *Mycoplasma gallisepticum* isolated from house finches (*Carpodacus mexicanus*) with conjunctivitis. *Avian Dis* **40**, 480-483.

Lin MY, Kleven SH (1982) Pathogenicity of two strains of Mycoplasma gallisepticum in turkeys. *Avian Dis* **26**, 360-364.

Lloyd-Smith JO, George D, Pepin KM*, et al.* (2009) Epidemic dynamics at the human-animal interface. *Science* **326**, 1362-1367.

Longdon B, Hadfield JD, Day JP*, et al.* (2015) The causes and consequences of changes in virulence following pathogen host shifts. *PLoS Pathog* **11**, e1004728.

Majumder S, Zappulla F, Silbart LK (2014) Mycoplasma gallisepticum lipid associated membrane proteins up-regulate inflammatory genes in chicken tracheal epithelial cells via TLR-2 ligation through an NF-kappaB dependent pathway. *PLoS One* **9**, e112796.

Majumder S, Zappulla F, Silbart LK (2016) Interaction of *Mycoplasma gallisepticum* with chicken tracheal and epithelial cells contributes to macrophage chemotaxis and activation. *Infection and Immunity* **84**, 266-274.

Mandel MJ, Wollenberg MS, Stabb EV, Visick KL, Ruby EG (2009) A single regulatory gene is sufficient to alter bacterial host range. *Nature* **458**, 215-218.

Mohammed J, Frasca S, Cecchini K*, et al.* (2007) Chemokine and cytokine gene expression profiles in chickens inoculated with *Mycoplasma gallisepticum* strains R-low or GT5. *Vaccine* **25**, 8611-8621.

Much P, Winner F, Stipkovits L, Rosengarten R, Citti C (2002) *Mycoplasma gallisepticum*: Influence of cell invasiveness on the outcome of experimental infection in chickens. *FEMS Immunol Med Microbiol* **34**, 181-186.

Nunoya T, Yagihashi T, Tajima M, Nagasawa Y (1995) Occurrence of keratoconjunctivitis apparently caused by *Mycoplasma gallisepticum* in layer chickens. *Vet Pathol* **32**, 11-18.

O'Connor RJ, Turner KS, Sander JE*, et al.* (1999) Pathogenic effects on domestic poultry of a *Mycoplasma gallisepticum* strain isolated from a wild house finch. *Avian Dis* **43**, 640-648.

Palmer MV (2007) Tuberculosis: a reemerging disease at the interface of domestic animals and wildlife. *Curr Top Microbiol Immunol* **315**, 195-215.

Rigaud T, Perrot-Minnot MJ, Brown MJ (2010) Parasite and host assemblages: embracing the reality will improve our knowledge of parasite transmission and virulence. *Proc Biol Sci* **277**, 3693-3702.

Roberts SR, Nolan PM, Hill GE (2001) Characterization of *Mycoplasma gallisepticum* infection in captive house finches (*Carpodacus mexicanus*) in 1998. *Avian Dis* **45**, 70-75.

Schrag SJ, Wiener P (1995) Emerging infectious disease: what are the relative roles of ecology and evolution? *Trends Ecol Evol* **10**, 319-324.

Staley M, Hill GE, Armbruster JW, Bonneaud C (*In prep*) Testing the role of host exposure in the host shift of an emerging bacterial pathogen.

Stallknecht DE, Luttrell MP, Fischer JR, Kleven SH (1998) Potential for transmission of the finch strain of *Mycoplasma gallisepticum* between house finches and chickens. *Avian Dis* **42**, 352-358.

Szczepanek SM, Tulman ER, Gorton TS*, et al.* (2010) Comparative genomic analyses of attenuated strains of *Mycoplasma gallisepticum*. *Infect Immun* **78**, 1760-1771.

Thomas L, Davidson M, McCluskey RT (1966) Studies of PPLO infection. I. The production of cerebral polyarteritis by *Mycoplasma gallisepticum* in turkeys; the neurotoxic property of the Mycoplasma. *J Exp Med* **123**, 897-912.

Tulman ER, Liao X, Szczepanek SM*, et al.* (2012) Extensive variation in surface lipoprotein gene content and genomic changes associated with virulence during evolution of a novel North American house finch epizootic strain of *Mycoplasma gallisepticum*. *Microbiology* **158**, 2073-2088.

Viana D, Comos M, McAdam PR*, et al.* (2015) A single natural nucleotide mutation alters bacterial pathogen host tropism. *Nat Genet* **47**, 361-366.

Vogl G, Plaickner A, Szathmary S*, et al.* (2008) *Mycoplasma gallisepticum* invades chicken erythrocytes during infection. *Infect Immun* **76**, 71-77.

Winner F, Rosengarten R, Citti C (2000) In vitro cell invasion of *Mycoplasma gallisepticum*. *Infect Immun* **68**, 4238-4244.

Woolhouse ME, Haydon DT, Antia R (2005) Emerging pathogens: the epidemiology and evolution of species jumps. *Trends Ecol Evol* **20**, 238-244.

Yagihashi T, Nunoya T, Sannai S, Tajima M (1992) Comparison of immunity induced with a Mycoplasma gallisepticum bacterin between high- and low-responder lines of chickens. *Avian Dis* **36**, 125-133.

Figures

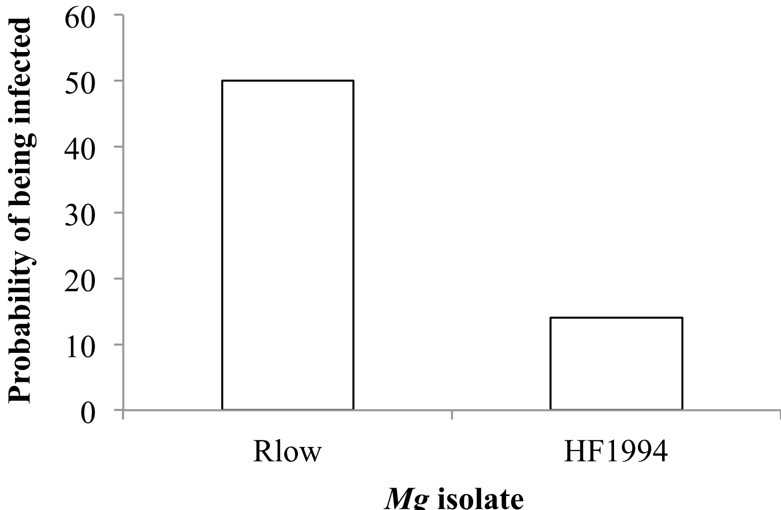

Figure 1. Probability of infection following inoculation with either a poultry strain of *Mg* (Rlow) or house finch epizootic-outbreak isolate (HF1994). We show the percent individuals found positive for Mg after PCR of either ocular swabs, conjunctivae or trachea on day 14 post-inoculation.

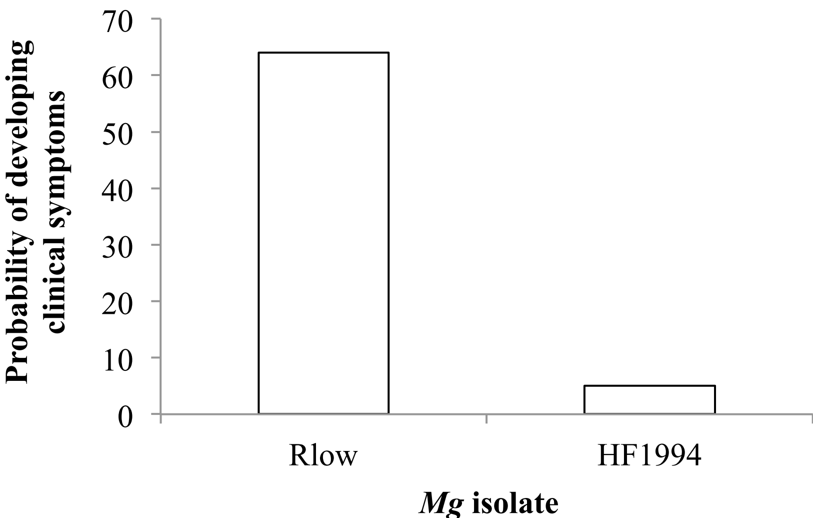

Figure 2. Probability of developing clinical symptoms following inoculation with either a poultry strain of *Mg* (Rlow) or house finch epizootic-outbreak isolate (HF1994). We show the percent individuals that developed visible clinical symptoms.

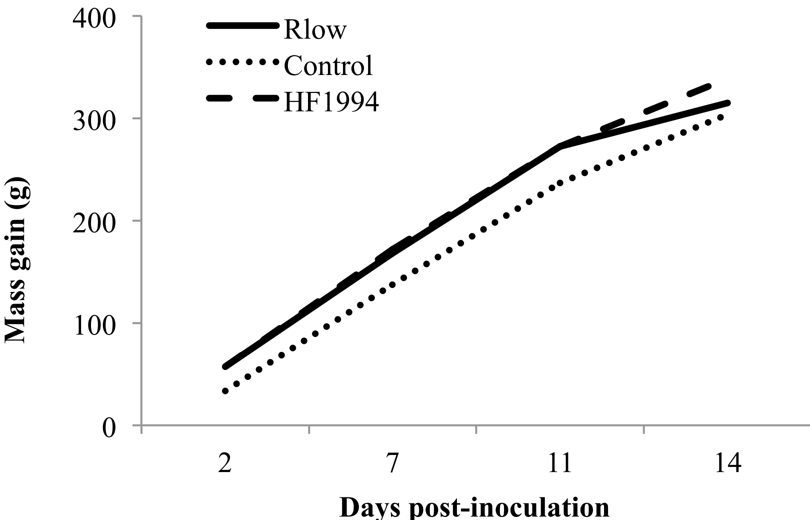

Figure 3. Mass gain (in g) over the course of the experiment for chicken inoculated with either chicken Mg (Rlow), house finch Mg (HF1994) or sham-inoculated controls. We show the predicted means of the model.