STUDY OF A NOVEL HOST-PARASITE RELATIONSHIP: *MYCOPLASMA GALLISEPTICUM* IN HOUSE FINCHES (*CARPODACUS MEXICANUS*)

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STUDY OF A NOVEL HOST-PARASITE RELATIONSHIP: *MYCOPLASMA GALLISEPTICUM* IN HOUSE FINCHES (*CARPODACUS MEXICANUS*)

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DISSERTATION ABSTRACT

STUDY OF A NOVEL HOST-PARSITE RELATIONSHIP: *MYCOPLASMA GALLISEPTICUM* IN HOUSE FINCHES (*CARPODACUS MEXICANUS*)

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Although host-parasite interactions are well recognized as dynamic interactions leading to the occurrence of emerging infectious disease, we know little about how host-parasite systems evolve. To better understand how these systems may evolve, I studied how the bacterium *Mycoplasma gallisepticum* (MG) and its new host, the house finch (*Carpodacus mexicanus*), have changed since the infection was first observed in the eastern population of the house finch in the mid-1990s.

To assess the evolution of MG in the house finch, the virulence of two isolates collected six years apart was examined. One isolate was collected in 1995 during the height of the epidemic, and the second isolate was collected in 2001, when the disease was less prevalent in the wild host population. For three of the four parameters of

virulence that were tested (Initial infective dose, severity of clinical disease one week post-exposure, and rate of recovery), the 2001 isolate was more virulent than the 1995 isolate. The results of my study indicate that the house finch MG strain has become significantly more adapted to survival in its newly colonized host.

The evolution of the host in response to infection with MG was also tested by comparing the response to MG infection using birds collected from an exposed population (Alabama) or from one of two naïve populations (California and Hawaii). While all three populations exhibited a range of responses to experimental infection with MG, in general, the results show that Alabama finches developed less severe clinical disease, and had a higher rate of recovery from infection than finches in the two naïve populations. These results support the theory that house finches in the eastern range have increased their resistance to MG.

To better understand the impact the house finch MG strain on other wild songbirds, birds from nine species were exposed to the house finch MG strain by ocular inoculation. All four species in the family Fringillidae and one non-fringillid were susceptible to infection and developed clinical disease. Three additional species were susceptible to infection with the house finch MG strain although they did not develop clinical disease. These results indicate that while Fringillids are more susceptible to the house finch MG strain, additional wild bird species are susceptible and may play a role in transmission.

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I. INTRODUCTION

Our world is teeming with rapidly evolving parasites and pathogens. When we also consider the rapid pace of global ecological change, emergence of new diseases, expansion of pathogen host-range, and reemergence of diseases are likely to be more frequent events. In order to cope with this ever increasing number of damaging and potentially lethal diseases it is important to understand how these novel host-parasite systems evolve. What changes occur in the parasite as is adapts to its new host? What changes does the host make to in response to novel infections? The traditional view on virulence evolution held that all pathogens should evolve to a state of benign existence in their hosts because destruction of their host would be detrimental to long-term pathogen survival (Smith, 1934; Zinsser, 1935; Dubos, 1965; Hoeprich, 1987). Therefore, virulence has been considered an artifact of recent or limited association between parasites and hosts. This view was supported by the many cases where a parasite infects an atypical host such as viruses that cause hemorrhagic fevers in humans and many emerging infections such as SARS or HIV that are highly virulent. However, many other highly virulent diseases such as smallpox or malaria have had a long history in human populations. The puzzling question then becomes, why do pathogens, which rely on their hosts for survival, cause disease and even death? Many evolutionary biologists believe there is no *a priori* reason that pathogens should evolve toward benign coexistence, and that virulence may evolve in either direction depending upon a number of

epidemiological and ecological factors such as mode of transmission and the ability of the parasite to persist in the host (Ewald, 1994; Levin, 1996).

In each generation of parasites there are mutations that lead to a variety of genotypes in the population. Natural selection favors the genotypes that are best able to pass their genes on to future generations. In evolutionary theory natural selection is acting on both within-host and between-host selection for genotypes (Ewald, 1983; Bull, 1994). Some evolutionary biologists believe that within-host selection may be the driving force behind the evolution of virulence (Sasaki and Iwasa, 1991; Anita et al., 1994; Bull, 1994; Nowak and May, 1994; Koella and Anita, 1995; Roode et al., 2005). Natural selection is a local phenomenon, and each host is a temporary island for the parasite. Within-host selection can change the frequency of parasitic genotypes competing within a given host. If virulence is associated with higher within-host replication than more highly virulent forms of the parasite will out-compete less virulent genotypes (Bremmerman and Pickering, 1983; Frank, 1992; Garnett an Anita, 1994). Alternately, it may be that less virulent genotypes can replicate more rapidly in the host, leading to reduced virulence (Bangham and Kirkwood, 1993).

Other scientists argue that the driving force behind the evolution of parasites is between-host selection. During between-host competition natural selection acts on the dynamics of parasite transmission between hosts, and parasite populations evolve to levels of virulence that maximize fitness where fitness is defined by rates of transmission and proportion of infected individuals (Bull, 1994; Levin, 1996). In the adaptive model, first proposed by Ewald (1983) suggests that virulence is a consequence of a parasite's efforts to maximize its fitness. Virulence is an unavoidable consequence of parasite

colonization in the host through the use of host tissues, metabolic waste, and/or toxins. Pathogens require extensive within-host replication to achieve maximum transmission to the next host, but increased replication causes increased virulence. The consequence of increased damage to the host is immobility and death, which may greatly reduce the parasites rate of transmission. The requirements for transmission provide a counterbalancing selection and ultimately, pathogens should evolve to the levels of virulence that maximize their fitness (Anderson and May, 1982; Frank, 1996; Ebert, 1999; Roode et al., 2005).

For the adaptive model route of transmission plays a vital role in determining the level of virulence for pathogens. In general, virulence is negatively correlated with host mobility, therefore the more a pathogen must rely on host mobility for transmission the less virulent it can afford to be. The best-studied example of this is the difference between parasites that transmit vertically and those that transmit horizontally. The greater the reliance on vertical transmission, the more a pathogen depends upon the mobility and fecundity of its host for its own fitness. For vertical transmission to occur the host must be able to reproduce and produce viable offspring that are also able to reproduce. This should lead to natural selection favoring less virulent genotypes. Several experimental studies have shown that even within a given host-parasite system the higher the rate of vertical transmission the lower the virulence (Bull et al., 1991; Messenger et al., 1999; Vizoso and Ebert, 2005).

Ewald (1983) performed a comparative study on the level of virulence (as measured by mortality) between vector-borne and nonvector-borne pathogens and found that vector-borne pathogens, which do not rely on host mobility for transmission, were

significantly more lethal to their hosts. Furthermore, the correlation between vector-borne diseases and virulence may also hold true for pathogens that use predators as vectors of disease. In both cases the parasite is not constrained by host mobility so the most rapidly replicating genotypes with have the greatest fitness even if they are highly virulent. The pathogen is often avirulent in the predator allowing complete mobilization to disperse the pathogen back into the environment or directly transmit the parasite to a new host (Ewald, 1994).

The high levels of virulence seen in some pathogens that would seem to rely heavily on host mobility for their transmission, such as respiratory pathogens, may be explained by their ability to persist outside of a host for extended periods of time. A pathogen that has the ability to "sit and wait" for another host it may not need to rely on the mobility of its current host for future transmission. Walther and Ewald (2004) looked at the correlation between durability of the pathogen in the external environment and levels of virulence in human respiratory diseases and found a positive correlation between virulence and durability of the parasite in the external environment in human respiratory diseases.

In some cases virulence or virulence factors may directly increase between-host transmission (Fenner et al., 1956; Bull, 1994). Some examples of this are coughing, sneezing, and conjunctivitis in respiratory pathogens, or open sores in directly transmitted pathogens. Some evolutionary biologists even caution that in some cases, virulence may be coincidental, and have no selective value for the parasite (Levin and Svanborg Eden, 1990).

Though details about many specific host-parasite systems are available, an understanding of the evolution of parasite virulence and host resistance remains a mystery (Bull, 1994; Ewald, 1994). Many of the above mentioned theories about the evolution of virulence remain only theories with little or no experimental support. With most host-parasite relationships it is difficult, if not impossible, to experimentally test for natural selection in the host and the pathogen separately. The house finch (*Carpodacus mexicanus*)- *Mycoplasma gallisepticum* system is an ideal model system to study the effects of evolving parasite virulence and host resistance when a parasite becomes an infectious disease in a previously unexposed host. Prior to 1994 *Mycoplasma gallisepticum* (MG) was typically found only in gallinaceous birds, most commonly, domestic poultry. The only songbirds previously in which MG has been detected were house sparrows (*Passer domesticus*) in India, and tree sparrows (*Passer montanus*) in Japan (Jain et al., 1971; Shimizu et al., 1979). In January 1994 during an unusually icy winter in the mid-Atlantic back-yard bird watchers in the Washington D.C. and Maryland area began to observe house finches at their feeders with swollen and crusty eyes. Over the next few months as the disease began to spread through the house finch population, hundreds of birds were submitted to wildlife rehabilitation centers. The causative agent was identified in June 1994 as MG, and by June 1996 less than 3 years after initial sightings mycoplasmal conjunctivitis had spread throughout the entire eastern range of the house finch (Fischer et al., 1997). Over the next few years, as the infection shifted from an epidemic to an endemic pathogen in the house finch population, rapid evolutionary changes in the relationship were clearly discernable at the phenotypic level. The prevalence of disease dropped from highs of around 60% in local populations to less

than 25%, and seasonal variation became more prominent (Ley, 1996; Nolan, 1998; Roberts et al., 2001; Faustino et al., 2004). The mortality of infected individuals in captivity also declined dramatically from nearly 100% (Nolan et al., 1998) to less than 5% (Roberts et al., 2001; Dhondt et al., 2003). This system provides several unique tools that allow experimental separation of evolutionary changes in the host from those in the pathogen. First, isolates of the house finch MG strain have been collected from infected finches in many locations throughout the eastern United States every year since the start of the epidemic. Second, the host is a readily available wild songbird that lives in close contact with humans and adapts readily to captivity. Third, the clinical disease, mycoplasmal conjunctivitis, is readily observed, allowing even amateur bird watchers to detect diseased birds at their feeders. Finally, there are two additional populations of house finches that have not been exposed to the pathogen, one a large genetically diverse population that genetically mimics the eastern population prior to the epidemic, and another small, bottlenecked population.

I had three main objectives in my study. The first was to determine in what direction the virulence of the house finch MG strain is evolving as it adapts to its new host. Is it evolving to a state of benign coexistence as the overall drop in prevalence and mortality may indicate? Second, to what extent has selection in the eastern range of the house finch increased the resistance of this population to this new pathogen? Finally, I examined the susceptibility of other wild bird species to infection with the house finch MG strain to better understand the potential role these species might play in the transmission of this infection.

To examine changes in the virulence of MG and resistance of the eastern house finches I performed cross-infection experiments between 2 isolates of the house finch MG strain, one collected at the height of the epidemic, and one collected at a time when the disease was less prevalent in the wild host population, and three populations of house finches, one, a part of the exposed eastern range, and two naïve populations. To determine the susceptibility of several wild bird species I experimentally exposed them to the house finch MG strain and examined their susceptibility to infection and the severity of disease.

II. LITERATURE REVIEW

The pathogen- *M. gallisepticum*:

Mycoplasmas are the smallest known living organisms (Morowitz and Tourtellotte, 1962; Baseman et al., 1997). They belong to the family *Mollicutes* (molis, soft; cutis, skin, in Latin) that includes all cell wall-less prokaryotes. The cytoplasm of mollicutes are encased solely by a plasma membrane composed of approximately 60% membrane proteins and 40% membrane lipids, 4 to 20% of which are sterols (Razin et al., 1998). Mycoplasmas also have an unusually high percentage of lipoproteins when compared to other eubacteria (Razin et al., 1998). Phylogenetic analysis of the mycoplasmas indicate that they are not the ancestral forms of current bacteria, but instead are evolutionarily advanced prokaryotes that have descended from low G + C content, Gram-positive bacteria and undergone degenerative evolution to streamline their genome size down to the minimum number of genes needed for survival and replication (Woese et al., 1980; Rogers et al., 1985). Genomes of the mycoplasmas are in a size range from less than 600kb (*Mycoplasma genitalium*; Fraser et al., 1995) to about 1400kb (*Mycoplasma mycoides*; Neimark and Kocan, 1990 and *Mycoplasma penetrans*; Sasaki et al., 2002), and may encode as few as 300 genes (Mushegian and Koonin, 1996; Hutchinson et al., 1999). The reduction in genome size, while biologically economical, has resulted in the complete loss of a cell wall and limited biosynthetic pathways,

reducing mycoplasmas to an obligate parasitic lifestyle that relies on the uptake of vital nutrients from their hosts.

Their minute size and lack of a cell wall baffled scientists about their identification for decades. A bovine pleuropneumonia agent (*Mycoplasma mycoides* sub. *mycoides*) was first cultivated in 1898 (Norcard and Roux, 1898; Manlioff, 1992). Due to their ability to pass through bacteria-blocking filters, resist penicillins and sulfonamides, and grow in tissue culture, they were misidentified as viruses until the 1930's when the true nature of viruses was better understood (Maniloff, 1992; Baseman et al., 1997). At about the same time scientists were beginning to realize mycoplasmas could not be viruses, Klieneberger (1935) isolated the L-form of *Streptobacillus moniliformis*. This led to the belief that mycoplasmas were L-forms living in conjunction with the bacteria. In the late 1950's advances in the understanding of cell wall synthesis (Lederberg and St. Clair, 1958) allowed the definition of L-forms as bacteria that had partially or completely lost their cell wall, and mycoplasmas were considered no more than stable L-forms of bacteria. Isolation of a variety of walled bacteria from mycoplasma cultures only confirmed this theory (Razin, 1969). Some scientists believed mycoplasmas were taxonomically autonomous organisms, and debates between scientists as to the true identity of mycoplasmas raged through the decade until DNA-DNA hybridization and G + C content analysis became available in the late 1960's. Genomic analysis finally established mycoplasmas as taxonomically separate bacteria (Maniloff, 1992).

As a consequence of their small genome and subsequent lack of many metabolic pathways, mycoplasmas are obligate parasites that must transfer biosynthetic precursors such as nucleotides, fatty acids, amino acids, and sterols from their host cells.

Fortunately, while relatively host specific, mycoplasmas have found a way to inhabit most other living organisms who can then provide these necessary precursors. They inhabit humans, mammals, reptiles, fish, birds, invertebrates, insects, plants, and fungi (Kirchhoff et al., 1987; Saglio and Whitcomb 1989; Stradtlander and Kirchhoff, 1990; Lee and Davis, 1992; Razin 1992; Brown, 2002). Adherence to host cells is a pre-requisite of colonization for all mollicutes, as they must form a close association to host cell membranes to facilitate the uptake of nutrients (Razin et al., 1998). Many mycoplasmas exhibit a pear-like structure with a polarized tip containing adhesin proteins and specialized organelles that mediate tight attachment to sialic acid residues of sialoglycoproteins on host cell surfaces (Stipkovits and Kempf, 1996). This is a complex process involving a number of accessory membrane proteins (Razin et al., 1998). Even though mycoplasmas must rely on a host to provide many of their nutrients, the vast majority of species are commensals, causing no disease in their host. A couple of species found in arthropods are symbiotic, and a few that inhabit humans, animals, and plants are parasitic (Tully, 1993; Razin et al., 1998). For humans and animals, mycoplasmas primarily adhere to the epithelial cells of the respiratory and urogenital tracts.

Due to their parasitic nature, mycoplasmas have developed the ability to avoid host immune defenses. One of the most common characteristics is their ability to avoid antibodies through antigenic variation or phenotypic switching. Surface proteins involved in cytadhesin, the most common targets of antibodies, are expressed from multigene families and can be modulated to escape immune detection (Baseman et al., 1997; Razin et al., 1998).

Fifteen species of mycoplasmas and one Ureaplasma have been isolated from chickens and turkeys (*M. cloacale, M. equifetale, M. gallinaceum, M. gallinarium, M. gallisepticum, M. gallopavonis, M. glycophilum, M. imitans, M. iners, M. iowae, M. laidlawii, M. lipofaciens, M. meleagridis, M. pullorum, M. synoviae, and Ureaplasma gallorale*; Stipkovits and Kempf, 1996). Of these, MG is the most economically important and is found worldwide (Kleven and Levisohn, 1996; Ley and Yoder, 1997). It mainly colonizes the respiratory system and urogenital tract of chickens and turkeys.

In 2003 the complete genome sequence of *Mycoplasma gallisepticum* strain R_{low} was published (Papaazisi et al., 2003). It contained 996,422 base pairs that contained 742 putative coding DNA sequences of which function has been assigned to 469. They found sequences coding for a family of five adhesin-like proteins, 133 membrane proteins that may bind sugar moieties for nutrient uptake or cytadherance, and approximately 75 proteins involved in membrane transport of biosynthetic molecules. As a consequence of the tight attachment they must form to host cells, MG usually exhibits a very narrow host range infecting mainly gallinaceous birds with sporadic reports of birds of other genera (Ley and Yoder, 1997; Razin et al., 1998; Levisohn and Kleven, 2000).

MG is highly contagious and typically infects a population with high morbidity, but low mortality. Mycoplasmas have the ability to switch their surface proteins hindering the attachments of antibodies to the cell surface. This antigenic variation of the MG surface proteins allows the bacteria to escape host immune systems (Levisohn et al., 1995, Gorton and Geary 1997; Glew et al., 2000) making it very difficult for the host to clear the infection on its own therefore infections are considered chronic (Stipkovits and Kempf, 1996). Infection with MG can cause a wide variety of clinical manifestations

from sub-clinical infection to chronic respiratory disease in chickens characterized by nasal exudate, coughing, sneezing, and dyspnea. Infectious sinusitis in turkeys is characterized by sinusitis with nasal exudate, coughing, sneezing, and mild conjunctivitis (Stripkovits and Kempf, 1996; Ley and Yoder, 1997). Lesions may include an excess of mucus, catarrhal exudate in the nares, sinuses, trachea, bronchi, lungs, and air sacs, air sac edema, and caseous exudate of the air sacs. In severe cases MG may enter the joints causing excess joint fluid, arthritis, and inflammation of tendovaginal sheaths, bursae, and synovial membranes. Even with sub-clinical infection, there may be a decrease in weight gain in broilers, mortality, condemnation of carcasses at the processing plant, or impaired feed conversion. In breeders and layers the disease may cause a reduction in egg yield and an increase in embryo mortality (Stipkovits and Kempf, 1996). The degree of severity is often associated with environmental stressors, age of the birds, or concurrent infection with other pathogens. In most cases, infection with MG is subclinical, and does not become apparent until the birds are stressed, either by poor environmental conditions such as cold weather, high ammonia levels, overcrowding, or egg-laying. Because of this, the incubation period can be as long as 38 weeks (Stipkovits and Kempf, 1996). However, MG is frequently part of a multi-factorial disease complex involving viruses (including vaccine strains) or bacteria such as *Escherichia coli* and *Haemophilus paragallinarium*, causing severe disease and mortality as high as 30% of the infected flock (Stipkovits and Kempf, 1996; Levisohn and Kleven, 2000).

MG is transmitted vertically from infected hens to their embryos, causing a 5-10% increase in embryo mortality. As eggs and day-old hatchlings are often shipped to separate facilities, infected chicks that hatch become reservoirs of infection that can

spread the disease great distances (Stipkovits and Kempf, 1996). MG is also transmitted by direct contact with infected individuals or contact with contaminated surfaces and people. Transmission is most likely to occur during the acute phase of infection that may last for weeks when high levels of bacteria are present in nasal discharge. Outbreaks can spread rapidly through entire flocks of poultry, although rates of transmission are dependent upon environmental conditions and density of susceptible birds. Distance of infected individuals from the reservoir was identified as the major risk factor for infection of swine with *M. hyopneumoniae* (Goodwin, 1985; Stark et al., 1992; Stark, 1998). One study found the presence of airborne *M. hyopneomoniae* in air samples taken from pig houses with acute respiratory disease and several studies have tested the resilience of MG to survival outside of a host. MG could be re-isolated from most inert surfaces after two days (three days from human hair), and up to as many as 14 days depending upon environmental conditions and the amount of organic waste present (Polak-Vogelzang, 1977; Yoder, 1991; Christensen et al., 1994). Therefore, even after depopulation of infected flocks, poorly cleaned and unsanitized surfaces may still be sources of contamination.

In most countries, control of MG is achieved through maintaining MG-free, commercial breeding stocks with routine monitoring and immediate slaughter of infected flocks. Antibodies to MG can be detected by a variety of commercially available systems such as haemagglutination inhibition assays (HI), serum plate agglutination (SPA) assays, or commercially available enzyme-linked immunosorbent assays (ELISA). The HI assay was traditionally the test of choice, taking advantage of MG's haemagglutinin properties to accurately titer antibody response, but in rare cases diagnostically significant titers are

not detectable until three or more weeks after infection (Kleven and Levisohn, 1996; APHIS, 1997; Levisohn et al., 2000). SPAs detect immunoglobulins of the M class (IgM), the earliest antibody response to infection, but they do not give very precise titers of antibody (Kleven 1975). ELISA kits vary between manufacturers, and non-specific reactions may occur (Glisson et al., 1984; Avakian and Kleven 1990; Kempf et al., 1994b). More recently, PCR has become an acceptable method of detection. It is rapid and can detect and differentiate mycoplasmas in a multiple pathogen infection (Kempf et al., 1994a).

For countries or farms where slaughter of infected individuals is not feasible, vaccines, either inactivated or live attenuated, are available (Whithear 1996). To control mycoplasmal infections there are several families of antibiotics that have been shown to be effective including tetracyclines, macrolides, quinolones (Baughn et al., 1978; Kleven et al., 1991; Yamamoto, 1991; Yoder, 1991). Dipping infected eggs into an antibiotic solution or injecting antibiotic into the airsac, can also decrease incidence of egg transmitted mycoplasmas (Hall et al., 1963; Ghazikhanian et al., 1980; Edson et l., 1987; Stipkovits et al., 1993).

The Host- *Carpodacus mexicanus*:

House finches are small, sexually dichromatic passerines in the family Fringillidae averaging 14cm in length and 20g in mass. Females are a drab brown-gray while males exhibit carotenoid-based plumage coloration from drab yellow to brilliant red on their crown, breast, and rump. House finches are one of the few species that has benefited from the interference and expansion of humans in North America. Before the

1900's house finches were in a range from Oregon down to Veracruz and Oaxaca Mexico, and from the Pacific coast to the western edge of the Great Plains (Long, 1981). The majority of their diet is made up of various seeds and they prefer to build cup-shaped nests in cavities (Hill, 1993). Their natural habitat is open grassland with scattered trees, canyons, or rock outcrops for nesting. They are not found in the interior of forests or in vast open grasslands or deserts, making the Great Plains to the west and the forests to the north natural population barriers (Bartholomew and Cade, 1956). As humans spread across the United States and converted the natural landscape into cities, towns, and suburbs ideal habitat for house finches was created with lawns, ornamental trees, and plenty of cavities for nest sites. Besides creating a vastly suitable habitat, back-yard bird feeding has increased tremendously. It is now estimated that one in three houses has a back-yard bird feeder (Ehrlich et al., 1988), providing house finches with an almost unlimited supply of food.

Besides the range expansion of the house finches in the western United States, the species was introduced directly to New York State. House finches make excellent cage birds, and the capture of house finches in California and Mexico and transportation of them east for sale in pet shops was very common in the late nineteenth and early twentieth century (Cant and Geis, 1961). The Migratory Bird Treaty Act in 1918 made this practice illegal, but finches were still being sold in pet shops until the 1940's. It is assumed that pet dealers released their captive finches into the "wild" in New York City to avoid prosecution by wildlife enforcement officers (Elliot and Arbib, 1953). Cant and Geis (1961) believe that with so many house finches being transported to the east and sold (it has been estimated at over 100,000 birds), it is highly likely many of these also

escaped or were set free by owners during this time. These birds, though relatively few in number, reproduced and spread rapidly south from Long Island to Florida, west to the Great plains, and north into Canada. As humans have continued to expand and create larger urban and suburban areas in the Great Plains, house finches have continued to spread eastward from the West, and westward from the East until their colonization across the United States is now almost complete.

House finches were also introduced to the Hawaiian Islands in the mid 1800's. Unfortunately, there was no documentation of their release and very little of the details is known. Based on information gathered from older inhabitants of the islands and knowledge of shipping practices, Grinnell (1911) hypothesized that the birds were being captured in the San Francisco Bay area, and transported on ships to Honolulu on the island of Oahu. There it is thought that they were deliberately released to colonize the island. Since the ships sailing at that time took weeks to reach the islands, it is believed only small numbers of birds were aboard the vessels, but with their high fecundity the finches spread rapidly to all of the other major islands where they have been in abundance since at least 1870 (Grinnell, 1911).

The number of birds who founded the eastern range and Hawaiian Islands is not known, but it has been estimated that as few as five (Eliot and Arbib, 1953), or more than 50 (Gant and Geis, 1961; Mundinger, 1975) finches founded the eastern population, and that fewer finches founded the Hawaiian population than the eastern population (Grinnell, 1911; Benner, 1991; Vazquez-Phillips, 1992). In general the smaller the number of individuals that founds a new population the greater the loss of genetic diversity. Founder effect can often lead to an increase in the population's susceptibility to pathogens. Two

masters' theses focused on analyzing differences in genetic diversity among and between the western, eastern, and Hawaiian populations of house finches. William Benner (1991) looked at mitochondrial DNA variation between finches from 14 locations throughout the bird's original range and introduced ranges. He found that the finches had little inter-population variability and that the eastern population of finches had maintained most of their haplotype diversity as compared to the western, parent population, but the Hawaiian population had not.

Manuel Vazquez-Phillips (1992) performed a similar study, but he looked at the allozymic variation of 31 loci in 568 finches from 16 different locations throughout the western, eastern, and Hawaiian ranges. When he examined general heterozygocity for these loci he found no significant differences either among or within the three ranges. When he compared the percentage of rare alleles, however, the Hawaiian finches had only 29.2% of the rare alleles found in the western finches, while the eastern finches had 51.8%. Both of these studies support the idea that while the Hawaiian house finch population did indeed undergo a genetic bottleneck, the eastern range of house finches was founded by a large enough number to avoid this and maintain most of the genetic diversity found in the western range of finches.

Even though the eastern range of house finches seems to have maintained most of the genetic diversity of their western progenitors, their natural behavior made them the perfect species for a population-wide epidemic. Every spring finches pair off and reproduce with each pair producing between two to three nests containing an average of four to five hatchlings each year. Young birds fledge and proceed to flock enmasse in early June to early August. These young, previously unexposed finches form flocks,

sometimes containing hundreds of birds. These flocks can often be seen congregating at backyard feeders. They will stay together usually through molt, a very energetically costly time, and then disperse great distances in all directions (Hill, 1993). Added to this, some eastern house finches have developed migratory behavior with many birds from the northeast and Great Lakes regions wintering in the south (Belthoff and Gauthreaux, 1991; Hamilton, 1991; Cant and Geis, 1961), potentially furthering the rapid spread of a contagious disease.

The host-pathogen system:

House finches with debilitating conjunctivitis were first reported in February 1994 in Maryland and Virginia, and the causative agent was subsequently identified as MG (Ley et al., 1996). By October mycoplasmal conjunctivitis was being reported by federal wildlife resource agencies in nine states from New York to North Carolina (Fischer et al., 1997). In November 1994 the Laboratory of Ornithology at Cornell University (Ithaca, New York) began the "House Finch Disease Survey". This laboratory, in conjunction with Bird Studies Canada at Long Point Bird Observatory (Port Rowan, Ontario, Canada), already had a very well established program called "Project Feeder Watch" in which volunteer participants across North America report birds observed at their feeders. Those "citizen scientists" residing in the eastern range of the house finch population were given monthly surveys to report observations of house finches and other species with conjunctivitis, but they were not asked to count of the number of infected finches. Because these eager volunteers were already in place and this project began early in the epidemic, it was possible to monitor the geographic spread of the disease across the

house finch population (Dhondt et al., 1998). By March 1995, 13 months after the first reported cases, the disease was observed in house finches from Ontario, Canada, south as far as Georgia, and west as far as Ohio and Kentucky. In the fall of 1995 the epidemic reached as far west as Indiana, Michigan, and Illinois. Mycoplasmal conjunctivitis was first seen in the Auburn University study population in late 1995 (Nolan et al., 1998). By June 1996 house finches in every state in their eastern range were observed with the disease (Fischer et al., 1997).

While disease prevalence is seasonal, peaking in the fall (corresponding to juvenile flocking behavior) and late winter (the time of greatest food shortage), the highest prevalence were seen in mid 1995 and 1996, both in new areas as the epidemic spread, and in the Mid-Atlantic where the disease began (Dhondt et al., 1998). During this time prevalence was estimated between 41-60% through both the House Finch Disease Survey and observations of banded populations (Dhondt et al., 1998; Nolan et al., 1998). In the Auburn Alabama population disease prevalence peaked in the summer of 1996 at 60% in both wild finches and captive flocks housed in an outdoor aviary (Nolan et al., 1998). Mortality of infected individuals was very high (100%) in captive flocks, (Nolan et al., 1998), leading to an estimated population decline of 21% by late 1996 (Sauer et al., 1997). Prior to the epidemic, house finches were more densely populated in Pennsylvania than Ontario Canada, and more densely populated in rural areas than urban areas. These differences in population density disappeared after the epidemic demonstrating a density-dependant decline (Hochachka and Dhondt, 2000). The Breeding Bird Survey, carried out by the Biological Resources Division of the United States Geological Survey, estimated the annual population growth from 1990 to 1994 at

almost 12%, dropping to 4.5% in 1994-1995 (Sauer et al., 1997). It is unclear if mortality of infected finches was due to the disease or starvation and predation. Mycoplasmas are not usually the primary cause of death in their hosts (Levisohn and Kleven, 2000), and volunteers in the House Finch Disease Survey reported diseased birds being taken by cats (Dhondt et al., 1998). However, Nolan et al. (1998) reported 100% mortality in captive flocks safe from predation and an unlimited supply of easily accessible food and water.

Within three years of the initial epidemic, prevalence of the disease had begun to decrease. The proportion of northeastern monitoring sites in the House Finch Disease Survey reporting at least one diseased bird sighting each month peaked at 59% in August 1995 and then declined each year to just 12% by July 1999 (Hartup et al., 2001). Prevalence among birds captured in Alabama dropped from 60% in the summer of 1996 to 23% in the summer of 1997 and 20% in the summer of 1998 (Nolan et al., 1998; Roberts et al., 2001b). Only 16% of house finches captured in Georgia from 2001-2003 showed signs of mycoplasmal disease (Altizer et al., 2004). In a second Georgia population 7% of finches captured from 1998-1999 were infected (Luttrell et al., 2001).

Early genetic analysis of 54 house finch isolates collected in 12 states from 1994 to 1999 using random amplification of polymorphic DNA (RAPD) or arbitrary primer PCR (AP-PCR) showed a single, unique profile, suggesting that a single strain of MG, distinct from chicken or turkey strains, was responsible for the entire epornitic (Ley et al., 1997; Luttrell et al., 1998; Hartup et al., 2000; Hartup et al., 2001). A more recent study, using polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) and nucleotide sequencing of a gene encoding a cytadhesin protein, showed genotypic differences among MG isolates collected from house finches (Pillai et al.,

2003). In the PCR-RFLP study, 55 isolates from 12 states were clustered into three distinct RFLP groups and 16 genotypes. Significantly, however, there were no discrete genetic changes from isolates collected early in the epidemic compared to those collected at later dates that would help explain the phenotypic changes in virulence seen in the wild (Pillai et al., 2003).

In poultry, MG is transmitted vertically to eggs as well as horizontally but to date, studies of the transmission of infection to eggs and nestlings have found that house finch parents do infect nestlings; there is no evidence of MG in eggs (Hartup et al., 1999; Nolan et al., 2004; Farmer, unpublished data). Infected nestlings do inhibit depressed growth rates and delayed dispersal in nestlings perhaps accounting for the overall decrease in body size seen after the epidemic (Nolan et al., 2004; Nolan et al, unpublished manuscript).

Two studies have looked at the effect of the house finch MG on chickens. Stallknecht et al. (1998) exposed chickens to infected house finches, by direct contact in the same cage, across a wire barrier, and to diseased finches housed 0.5m away. After 10 weeks of direct contact with infected finches, 80% of chickens seroconverted, and MG was cultured from 30%. Isolates collected were identified as the house finch MG by arbitrary primed PCR. MG was not detected in any of the chickens exposed to infected finches across the wire barrier or from 0.5m away. O'Connor et al. (1999) compared chickens and turkeys exposed to either the house finch MG or the R-strain of MG by aerosolized inoculums. They found that while they could detect the house finch MG in chickens and turkey at 28 days post exposure, no clinical signs of disease occurred, and

histologic lesions were milder in comparison to those caused by infection with the R strain.

Conjunctivitis has also been reported from over 30 additional passerine species (Hartup et al., 2000, 2001; Mikaelian et al., 2001). However, MG infection has not been confirmed as the causative agent in most of these reported observations. In field studies on wild birds antibodies to MG have been detected by agglutination assays in over 20 wild bird species, including ten of the species in which conjunctivitis has been reported (Hartup et al., 2000; Luttrell et al., 2001). Although this suggests natural infection, the specificity of these serologic tests in wild bird species other than finches is unclear. MG has been confirmed by PCR or culture in only six additional wild avian species: American goldfinch *(Carduelis tristi*s), purple finch *(Carpodacus purpureu*s), eastern tufted titmouse *(Baeolophus bicoo*r), pine grosbeak *(Pinicola enucleato*r), and evening grosbeak *(Coccothraustes vespertinu*s), and one blue jay (*Cyanocitta cristata*; Fischer et al., 1997; Hartup et al., 2000; Luttrell et al., 2001; Mikaelian et al., 2001). Although house finches and American goldfinches aggregate at feeders and are often seen feeding side-by-side, American goldfinches are far less frequently observed with conjunctivitis (Hartup et al., 2001; Luttrell et al., 2001).

III. SUSCEPTIBILITY OF A NAIVE POPULATION OF HOUSE FINCHES TO *MYCOPLASMA GALLISEPTICUM*

ABSTRACT. Since 1994 an epidemic of mycoplasmal conjunctivitis has spread throughout the eastern population of the house finch (*Carpodacus mexicanus*), leading to a significant decline in this population. The infection has not yet been reported from house finch populations west of the Great Plains. I hypothesized that the western population, like the eastern population, is susceptible to the infection, and I tested this hypothesis by experimentally infecting house finches from Missoula, Montana (USA) with the house finch strain of *Mycoplasma gallisepticum* (MG). I compared the response of finches from Montana infected with MG to that of finches from Auburn, Alabama (USA) (October 1999- February 2000). Fifteen house finches from Montana were shipped to Auburn and quarantined for 6 wk at the Auburn University aviary. All birds were negative for antibodies to MG when tested by the serum plate agglutination assay and MG could not be detected in any bird by polymerase chain reaction. I tested two methods of inoculation, ocular inoculation and contact exposure to an infected finch. Seven house finches from Montana and four house finches from Alabama were infected by bilateral ocular inoculation with 20ul of a culture containing $1X10^6$ color changing units of the house finch strain of MG. The remaining eight house finches from Montana were co-housed with a house finch from Alabama exhibiting mycoplasmal conjunctivitis.

After exposure to the pathogen, all house finches became infected, regardless of origin or method of exposure, and all developed conjunctivitis. All birds seroconverted, and evidence of infection could be detected in every bird at some point during the course of disease. These results suggest that house finches from the western United States are highly susceptible to infection with the house finch strain of MG.

Key words: Carpodacus mexicanus, conjunctivitis, house finch, *Mycoplasma gallisepticum*, western house finch

INTRODUCTION

In February 1994 an outbreak of conjunctivitis was observed in house finches (*Carpodacus mexicanus*) in suburban Washington D.C. (USA; Ley et al., 1996; Luttrell et al., 1996). Mycoplasmas were isolated from lesions of affected birds and subsequently identified as a unique strain of *Mycoplasma gallisepticum* (MG), a pathogen not previously isolated from passerines in North America (Ley et al., 1996). From the point of initial detection the disease spread through the entire eastern population of house finches (Fischer et al., 1997; Dhondt et al., 1998). In the early years of the epidemic, both the prevalence of the clinical disease and mortality were high (Sauer et al., 1997; Nolan et al., 1998). In recent years, mortality and prevalence of the disease has declined (Hartup et al., 2000; Roberts et al., 2001b).

House finches are not native to eastern North America. House finches originating from coastal California were introduced to Long Island, New York in 1940 (Elliot and Aribib, 1953), and from there the birds have spread throughout the eastern United States

and into Canada (Hill, 1993). The number of birds that founded the eastern house finch population is unknown, but authors speculate that 50 or more individuals founded the eastern population (Cant, 1962; Mundinger, 1975; Hill, 1993). Although the eastern population originated from a relatively small number of birds, it appears that most of the genetic diversity of the parent population has been retained (Vazquez-Phillips, 1992). Populations in the Great Plains, which divides the native western and introduced eastern populations, are sparse due to lack of suitable habitat. Gene flow and disease transmission between the eastern and western populations of house finches appears to be low (Hill, 1993) and there have been no published reports of mycoplasmal conjunctivitis west of the 100th meridian.

Many basic questions regarding this new host-pathogen relationship remain to be answered. The objective of this study was to test the susceptibility of western house finches to the house finch MG strain. I infected western house finches from Missoula, Montana with an isolate of the house finch MG strain and compared their response to that of eastern house finches from Auburn, Alabama. I also compared two routes of infection: bilateral ocular inoculation and direct exposure to an infected finch.

MATERIALS AND METHODS

House finches were captured in Missoula, Montana (46^{o}52'N, 114^{o}00'W) in October 1999 and transported to the Auburn University campus (32°35'N, 85°28'W). The birds were trapped using wire-mesh basket traps under permits from Montana Department of Fish, Wildlife, and Parks (Helena, Montana, USA; No. 1456) and a federal collecting permit (MB784373-2). All procedures involving live animals were reviewed

and approved by the Auburn University Institutional Animal Care and Use Committee (PRN# 0303R2249). Upon arrival the house finches were banded and divided into three flocks of seven or eight birds. Each flock was housed separately in an indoor, temperature-controlled room (1.6m x 2.3 x 2.6h) with natural light and maintained on a diet of sunflower seed, red and white millet, and water ad libitum with sufficient grit. The water was supplemented with Premium Multi-Drops high potency multivitamins for caged birds (8 in 1 Pet Products, Inc., Hauppauge, New York, USA.). Four house finches from Auburn, Alabama were caught and housed in a similar manner to the Montana house finches under a permit from the Alabama Department of Conservation (Montgomery, Alabama, USA; No. 12). All finches used in this study were identified as 1999 hatch-year birds based on plumage.

To prevent transmission of infection between flocks a quarantine area was established around the door to each room. Investigators wore gloves and disposable booties when entering the rooms or handling the birds. All dishes were soaked in a 10% bleach solution, and separate nets were used to capture each flock. All four flocks were quarantined for 6 wk prior to infection to monitor for any diseases. At the end of the quarantine period, blood was collected from all 27 birds for serology as previously described (Roberts et al., 2001b). The blood was tested for antibodies to MG with a commercial serum plate agglutination (SPA) assay (Luttrell et al., 1996) (Intervet Inc., Millsboro, Delaware, USA). After 2 min the extent of agglutination was scored on a scale of 0 to 4, with a score of 2 or greater considered positive. Birds also were tested for the presence of MG by polymerase chain reaction (PCR). Samples for PCR analysis were obtained by gently swabbing the choanal cleft using a microtip swab (Becton Dickinson

and Co., Sparks, Maryland, USA). DNA extraction and PCR amplification of a 185-bp fragment using MG-specific primers (LTI, Gaithersburg, Maryland, USA) was preformed as described previously (Lauerman, 1998; Roberts et al., 2001b).

The house finches were exposed to MG by one of two methods. One group of 11 house finches, including the four from Alabama, were inoculated with a 72-hr broth culture of house finch MG, grown in SP4 broth, via a bilateral ocular route for a total dose of 20 ul/bird. Serial dilution of the broth culture determined each dose contained $1X10^6$ color changing units per ml of MG (generously provided by P. Luttrell, Southeastern Cooperative Wildlife Disease Study, The University of Georgia, Athens, Georgia, USA). The isolate was obtained in Clark County, Georgia in November 1995 and had undergone five passages before being introduced into the birds. A second group of eight house finches from Montana were exposed to MG by co-housing them in a room with a house finch, caught in Auburn, Alabama, exhibiting mycoplasmal conjunctivitis. MG was confirmed in the Alabama house finch by SPA, culture (see below), and PCR. The remaining eight house finches from Montana were inoculated with sterile SP4 broth and served as a negative control.

The finches were monitored daily for signs of disease. Once each week for 12 wk the finches were captured and scored for conjunctivitis in each eye using a scale from 0-4 with one being minimal signs of the disease and four complete blindness due to swelling of the conjunctiva. During weekly capture birds were bled for serology and swabbed for PCR analysis. Three attempts at isolation of the MG were made during the study. The infected finch from Alabama introduced into the flock of birds from Montana, one of the finches from Montana in that flock, and one finch from Montana inoculated with the

isolate from 1995 were swabbed as described above and the swab was placed into a SP4 broth tube pre-warmed to 37°C. A blind 1:10 passage was made 24 hr following initial culture. Broth cultures were incubated at 37°C for 5 wk or until a phenol-red- indicated color change occurred at which time the culture was tested for the presence of MG by PCR.

Results for finches are given in average days ± one standard deviation. Differences in incubation period and duration of illness between house finches from Montana and house finches from Alabama directly inoculated with an MG isolate were evaluated with a two-tailed Mann-Whitney U test. This same test was also used to evaluate the difference between house finches from Montana inoculated directly and those exposed to an infected bird. Differences in mortality between these flocks were evaluated with a Chi-squared (χ^2) test. All statistics were done using Statsview 5.0.

RESULTS

Prior to infection, all birds were healthy. None of the house finches had antibodies to MG, and MG was not detected by PCR. After exposure to MG all birds became infected and developed conjunctivitis, regardless of the method of exposure or the geographic origin of the finches. All of the birds seroconverted, and MG was detected by PCR in every bird at least once during the course of the disease. All three attempts at isolation of MG from infected finches exhibited a color change indicating growth and MG was confirmed by PCR. The eight control finches never exhibited signs of disease and MG was not detected by serology or PCR at any time during in these birds.

Following ocular inoculation with MG all the finches rapidly developed conjunctivitis, but the incubation period in the finches from Alabama was an average of 5.25 ± 1 days, significantly longer than the incubation period of the finches from Montana (3.71 ±1.1 days) ($Z = -1.97$, $p= 0.049$; Fig. 1A). All of these finches exhibited moderate to severe unilateral or bilateral conjunctivitis (score of 2-3) with some birds experiencing complete blindness in one or both eyes (score of 4). Although six of the seven finches from Montana and all four finches from Alabama died during the study, the house finches from Alabama survived an average of 58 ± 9.9 days from onset of disease, significantly longer than the house finches from Montana who only survived an average of 35 ± 11.8 days after onset of disease ($Z= -2.15$, $p = 0.03$; Fig. 1B).

When the finches from Montana were exposed to MG by co-housing them with a naturally infected finch from Alabama we observed a significantly longer (20.6± 8.1 days) and more variable time period until all of finches in the flock showed clinical disease when compared to the finches from Montana exposed to MG by ocular inoculation ($Z = -3.28$, $p=0.001$; Fig. 1A). Regardless of mode of exposure we observed no differences between these two flocks in the severity of disease, duration of illness ($Z = - 0.13$, $P = 0.90$; Fig. 1B), or mortality rate ($\chi^2 = 0.01$, d.f. =1, $P = 0.94$).

DISCUSSION

These results indicate that finches from a population in Montana, part of the western population of house finches, are highly susceptible to infection with the house finch MG strain. All 15 house finches captured in Montana and experimentally infected with MG by direct exposure to MG or by being housed with an infected finch developed

clinical disease. I was able to detect MG by PCR and antibody production in their blood serum by SPA. These observations suggest that the large western population of house finches may be susceptible to the house finch MG strain, and if MG spread to the western population it would spread rapidly causing an epidemic similar to the eastern population killing millions of house finches.

I also observed differences in response to infection between finches from Montana and finches from Alabama. Previous published reports of both wild and captive finches indicate a changing relationship between MG and the house finch (Luttrell et al., 1996; Nolan et al., 1997; Roberts et al., 2001a; Roberts et al., 2001b). One reason for this change may be an increased resistance in the house finch to MG. Although all four finches from Alabama died during the course of the study they all survived significantly longer than the finches from Montana who died. This may suggest an increase in resistance to the pathogen in the exposed population when compared to the response of the naïve western population.

I also wanted to compare the response in birds to different modes of exposure. This study demonstrates that finches are susceptible to infection with MG by either co-housing the birds with an infected finch or by direct ocular inoculation and their response to infection is the same regardless of the method used.

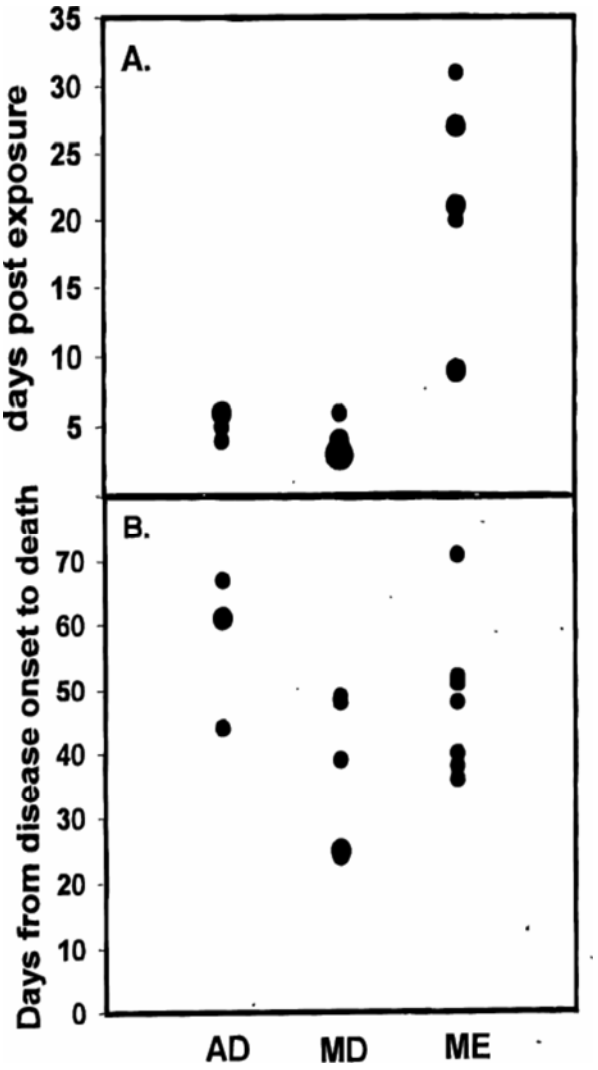

Figure 1. Differences in (A) time period until all finches demonstrate clinical disease and (B) duration of illness between three flocks of house finches. AD = finches from Alabama inoculated directly, MD = finches from Montana inoculated directly, and ME = finches from Montana exposed to a naturally infected finch.

IV. CHANGING RATES OF INFECTION OF HOUSE FINCHES BY *MYCOPLASMA GALLISEPTICUM*: BETTER HOST RESISTANCE OR REDUCED PATHOGEN VIRULENCE?

ABSTRACT. Understanding the relationships between hosts and parasites has far reaching applications from vaccine development to protecting biodiversity. Though many details about host-parasite systems are available our understanding of the evolution of parasite virulence and host resistance is limited. For most systems it is difficult to experimentally test for pathogen and host evolution separately. To better understand how emerging diseases may evolve, I independently tested for changes in host resistance and parasite virulence in the novel house finch (*Carpodacus mexicanus*)- *Mycoplasma gallisepticum* system. To assess the evolution of MG in the house finch, the virulence of two isolates collected six years apart was examined. For three of the four parameters of virulence that were tested (Initial infective dose, severity of clinical disease one week post-exposure, and rate of recovery), the isolate collected at a time when the disease was less prevalent in the wild host population was more virulent than the isolate collected at the height of the epidemic. To assess changes in resistance of house finches I compared the response to infection of birds collected from an exposed population (Alabama) or from one of two naïve populations (California and Hawaii). In general, the results show that Alabama finches developed less severe clinical disease, and had a

higher rate of recovery from infection than finches in the two naïve populations. My results suggest that as the house finch MG strain is adapting to its new host virulence is increasing. At the same time, house finches in the eastern range have increased their resistance to MG.

Key words: Carpodacus mexicanus, dose-response, evolution, host-parasite, house finch, *Mycoplasma gallisepticum*

INTRODUCTION

The co-evolution of a novel host-parasite relationship can involve rapid evolutionary changes of both the parasite and the host. Parasites must adapt quickly to the selective pressure of the host's immune response while competing for space and resources (de Campos et al., 1993; Bull, 1994; Goulder et al., 1997; McMichael, 1998; deRoode et al., 2005). In turn, parasites can have enormous impacts on their vertebrate hosts, providing intense selective pressures as they reduce the fitness of individuals (e.g., Hamilton and Zuk, 1982, Hudson et al., 1998). The consequences of this selection can sometimes be observed as changes in population-wide susceptibility to infection (e.g., Fenner and Ratcliffe, 1965; 1986, Warner 1968; Van Riper III et al. 1986; Lenghaus et al., 1994) or in the virulence of a pathogen (Ross, 1982; Messenger et al., 1999 deRoode et al., 2005; Vizoso and Ebert, 2005). The specific genes involved in parasite resistance can potentially be detected in comparisons of the genetic profiles of diseased and non-diseased populations (e.g., Hill et al., 1991; Wang et al., 2006).

There are now several well-studied examples of the co-evolutionary interactions between parasites and vertebrate hosts (e.g. Fenner and Ratcliffe, 1965; Anderson and May, 1982; Ewald, 1994), but many of the most detailed examples of such interactions come from studies on human pathogens (reviewed in Ewald, 1994). Given the increasing relevance of host parasite co-evolution to a diversity of disciplines from vaccine development to conservation biology (Ebert, 1998; Kaiser; 1998), there is a need for more data from natural populations, particularly non-mammalian vertebrates. Moreover, the evolutionary dynamics of a host infected by a novel pathogen is of particular interest, both to assess theories on how such interactions evolve (Anderson and May, 1982; Levin, 1996; Ewald, 1994; Walther and Ewald, 2004), and to develop strategies for control and prevention of emerging infectious diseases in both humans and non-human species(reviewed in Lashley and Durham, 2002; Williams et al., 2002,).

Here I experimentally tested the change in virulence of a bacterial pathogen, MG, and the change in resistance of its recently colonized vertebrate host, the house finch (*Carpodacus mexicanus*) between initial colonization and seven years into the epornitic.

MG is a well-characterized and economically costly disease of domestic poultry that causes upper respiratory distress, weight loss, and reduced egg yields (Ley and Yoder, 1997). Prior to February 1994, when it was first isolated in Maryland from house finches with conjunctivitis, this pathogen was essentially unknown in songbirds (Ley et al., 1996). By October 1994, mycoplasmal conjunctivitis had been reported in house finches in nine states and by 1996 MG had spread through the entire eastern range of the house finch (Fischer et al., 1997). In house finches, infection with MG is characterized by

conjunctivitis and debilitation, sometimes accompanied by mild to severe ocular swelling, nasal and ocular discharge.

Despite the brief time since MG emerged as an infectious agent in the eastern population of house finches, changes in the host-parasite relationship are clearly discernable in observations of the proportion of clinically diseased birds. As MG spread throughout the eastern range of the house finches from 1994 to 1996 initial rates of infection were estimated at 41-60% through both the study of banded populations (Nolan et al., 1998), and by a national network of amateur bird-feeder watchers (Dhondt et al., 1998). Mortality among infected individuals during this initial infection period was high, leading to an estimated 21% reduction in the affected population (Sauer et al., 1997; Nolan et al., 1998). Within three years of the initial epidemic, however, overall prevalence of the disease had begun to drop and strong seasonal variations became apparent. The proportion of northeastern monitoring sites in the House Finch Disease Survey reporting at least one diseased bird sighting each month peaked at 59% in August 1995 and then declined each year to just 12% by July 1999 (Hartup et al., 2001). Prevalence among birds captured in an Auburn Alabama population showed a similar decline. Prevalence of MG dropped from 60% in the summer of 1996 to 23% and 20% in the summers of 1997 and 1998 respectively (Nolan et al., 1998; Roberts et al., 2001b). Only 7% of finches captured around poultry facilities in Georgia from 1998-1999 were infected (Luttrell et al., 2001). In a study conducted on a population of house finches in Atlanta, Georgia 888 finches were captured between August 2001 and December 2003. Monthly prevalence fluctuated from as high as 60% in October 2001 to 0% in March-

May 2002. Peaks were observed each year in September-October, coinciding with the high numbers of hatch-year birds and fall molt.

The observation that the disease prevalence declined rapidly after the initial colonization of house finches raises several fundamental questions. What caused the rapid and dramatic decline in the incidence of disease? Did the pathogen evolve reduced virulence? Did the birds evolve increased resistance? Because the interactions of house finches and MG have been closely observed since the beginning of the epornitic, this is an excellent system in which to test these basic questions.

Early genetic analysis of 54 house finch isolates collected in twelve states from 1994 to 1999 using random amplification of polymorphic DNA (RAPD) or arbitrary primer PCR (AP-PCR) showed a single, unique profile, suggesting that a single strain of MG, distinct from chicken or turkey strains, was responsible for the epornitic (Ley et al., 1997; Luttrell et al., 1998; Hartup et al., 2000; Hartup et al., 2001). A more recent study, using polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) and nucleotide sequencing of a cytadhesin protein reveals genotypic differences among MG isolates collected from house finches (Pillai et al., 2003). In the PCR-RFLP study, fifty-five isolates from twelve states were clustered into three distinct RFLP groups and 16 genotypes. Significantly, however, there were no discrete genetic changes from isolates collected early in the epidemic compared to those collected at later dates that would help explain the phenotypic changes in virulence seen in the wild (Pillai et al., 2003).

The objectives of this study were first to experimentally test the virulence of an isolate of MG collected during the second year of the epidemic when a high proportion of

wild birds were infected (1995) and an isolate collected six years later when a lower proportion of finches were infected (2001). House finches randomly drawn from single populations were exposed to the two MG isolates, thus randomizing the effect of avian genotype on disease response. I predicted that if the decrease in prevalence of disease seen in the wild was due mainly to changes in the bacteria, the isolate collected during the height of the epidemic should exhibit a higher virulence than the isolate collected later.

The second objective was to experimentally test the response of populations of house finches that either had an eight-year history of exposure to MG (i.e. individuals from the eastern U. S. population) or populations of finches that had never been exposed to MG. House finches are native to western North America. House finches from coastal California were released in New York City around 1940 (Elliot and Aribib, 1953; Hill, 2002), and from this introduction house finches have spread throughout eastern North America. Populations along the Great Plains are sparse, creating a barrier of gene flow and disease transmission between the western and eastern populations (Hill, 2002). House finches of unknown origin were also introduced to the Hawaiian Islands before 1870 (Grinnell, 1911). There are no records of the Hawaiian introduction, but it has been assumed that only a few birds founded the population (Badyaev and Hill, 2000).

House finches in both western North America and Hawaii have had no exposure to the house finch MG strain since its emergence in 1994, and there are no historical records of this disease in either of these populations. I experimentally exposed house finches from Alabama, California, and Hawaii to the two MG isolates and closely monitored their response. I predicted that if eastern finches evolved increased resistance

in response to the epidemic then Alabama finches should show less severe effects of experimental exposure than western or Hawaiian house finches.

MATERIALS AND METHODS

In May and June 2002, 98 house finches in Auburn, Alabama, 74 house finches at Pohakuloa Training Area on Hawaii Island Hawaii and 97 house finches in San Jose California were captured. The birds from Hawaii and California were air freighted to Auburn Alabama. All birds were given an individual metal leg band for identification, and housed in doors in pairs in cages $0.5m^3$ at the Auburn University Aviary. The birds were given water and a pellet finch food *ad libitum*, and were maintained on 13 hours of light at 25°C before and during the study. All finches appeared healthy and free of avian pox, a virus common among passerines. To ensure Alabama finches had not previously been infected with MG only hatch-year birds we caught and subsequently tested for MG and antibodies to MG the day they were captured. To test for MG, the choanal cleft of each bird was swabbed using a micro tip swab (Becton Dickinson and Co., Sparks, Maryland, USA). The extracted DNA was then amplified by PCR using MG-specific primers (Roberts et al., 2001b). To test for antibodies to MG, 100uL of blood was collected by veinipuncture from the brachial vein and the blood plasma was assayed for antibodies to MG using a commercial serum plate agglutination assay (SPA; Intervet Inc., Millsboro, Delaware) as described in Farmer et al,(2002). All of the finches were negative for MG and MG antibodies.

Two weeks prior to inoculation, birds from each population were randomly selected, and moved to cages in our infection rooms with negative air pressure systems.

To avoid any chance of cross-contamination between the two isolates finches with the 2001 isolate were inoculated in August 2002. After completion of the 12-week study the rooms were emptied and the cages and rooms were power sprayed to remove organic waste, then sprayed with 10% Clorox. The rooms and cages were then sprayed again with 10% Clorox one week before the second set of finches was moved in. After a two-week acclimation period the finches were inoculated with the 1995 isolate of MG in June 2003.

Two isolates, collected at different times during the epornitic, were used in this study. The 1995 isolate, collected from an infected house finch in Clark County Georgia in November 1995, was provided by P. Luttrell. This isolate was eight passages from original culture. The second isolate was collected from an infected house finch in Lee County Alabama in September 2001 and was three passages removed from the original isolate. All stock cultures were grown in SP4 medium. Large-scale stocks were grown at 37°C, aliquoted, and stored in 25% glycerol at –80°C. The stocks were titered using a color-changing assay (Cherry and Taylor-Robinson, 1970).

Experimental design. Birds from all three populations were tested against either the 1995 or the 2001 isolate of MG. An aliquot of the isolate was warmed to 37°C and diluted to the proper dose. Birds were inoculated by dropping 0.01ml of culture into each eye as previously described (Farmer et al., 2002) for a total of 0.02mls. The isolates were then taken back to the lab and re-titered. Each back-titer was performed in triplicate using the same amount of MG as was used to inoculate the finches (0.02ml) to ensure birds were given the intended dose of MG.

Birds inoculated with the 1995 isolate were inoculated with one of three doses of MG: a low dose of MG (2 CCUs; Auburn n=18, California n=19, Hawaii n=15), a

medium dose (200 CCUs; Auburn n=19, California n=20, Hawaii n=11), or a high dose (20,000 CCUs; Auburn n=20, California n=20, Hawaii n=14). Birds from all three populations inoculated with the 2001 isolate were inoculated with either the low (Auburn n=20, California n=18, Hawaii n=16), or medium (Auburn n=19, California n=18, Hawaii n=16) dose of MG. Two birds from each population were sham inoculated with the same amount of sterile SP4 media.

Each week post exposure the birds were captured and each eye was scored for degree of conjunctivitis on a scale of one to four (Farmer et al., 2002), and their choanal cleft was swabbed for PCR detection of the MG. Swabs were then stored at –20°C until extracted (Roberts et al, 2001b) and analyzed at the end of the study. For the first three to six weeks 100uL of blood was also collected to test for antibodies to MG. Collected serum was stored a 4°C and SPAs run every other week blind to the treatment group. At weeks one and three the choanal swab was swirled in warmed SP4 medium to attempt to culture the MG. Cultures were incubated for five weeks or until a color change indicated growth. All cultures were extracted and confirmed as MG by PCR. Sampling for each treatment group lasted 12 weeks or until all birds in that group were negative for all parameters of infection for two consecutive weeks.

Twelve weeks post-inoculation all of the finches were euthanized by CO_2 asphyxiation. For finches selected for necropsy the feathers were wetted with a soap solution and a slit was made in the skin at the keel. The skin and feathers were then removed from the ventral side of the beak to the lower abdomen and the keel was removed. After gross examination the trachea and lungs were removed and placed in 10%

neutral buffered formalin for histopathology. Sections were stained, and examined under light microscopy for any changes.

Data analysis. The ID_{50} of each isolate was calculated using the Reed and Muench method as previously described (Batson, 1956) and the data compared in a one sample, two tailed T-test. To identify differences in the severity of disease between the two isolates and between the three populations of finches one week after inoculation, Polytomous Universal model (PLUM) ordinal logistic regressions were performed using the week-one eye scores of infected birds. Isolate and population were the independent variables and there was no interaction effect between them ($n = 107$, $R^2 = .18$, $p = .487$). To assess differences in severity of disease we compared the highest eye score for each finch during the twelve-week study. Similar regressions were run, and again, there was no interaction effect between population and isolate ($n = 107$, $R^2 = .09$, $p = .436$). To examine differences in recovery from infection with MG each finch was given a score of 0 if they had cleared the infection (negative for MG by PCR and had minimal to no clinical disease by the end of the twelve weeks), and a 1 if they had not cleared the infection (positive for MG by PCR and moderate to severe clinical signs) or died while infected. There were no birds that did not fit into one of the two categories, however, 11 finches were excluded from this analysis because they died of causes other than infection with MG during the study. To examine whether population and/or location could predict if MG caused lesions in the trachea or lung or caused death in infected birds, birds were assigned a 0 if no lesions were found or they survived or a 1 if lesions were present or they died. Logistic regressions were run for recovery, lung, trachea, and death. Isolate and population were set as the independent variables, and population was classified as

categorical with Alabama set as the reference group. There were no interaction effects between population and isolate in any of these regressions (n= 97, R^2= .17, p= .532; n= 33, R^2 = .21, p= .99; n= 33, R^2= .18, p= .99; n= 96, R^2= .16, p= 1.0) respectively. All statistics were performed using SPSS v.14.

RESULTS

To evaluate differences in the virulence of the two isolates and the resistance of the three populations to infection I compared the ID_{50}, severity of disease one week after inoculation, overall severity of disease, recovery rate, and histopathology of the infected house finches. House finches from all populations required a100 times greater dose of 1995 isolate to develop clinical infection compared to the 2001 isolate (Table 1). Twenty-two percent of the 50 birds from all three populations exposed to 200 CCUs of the 1995 isolate seroconverted, indicating that they had been infected, but only 2 of the birds developed mild, short-lived conjunctivitis, and I was unable to isolate the bacteria or detect MG by PCR from any of the exposed birds. However, 100% of the 53 birds exposed to the same dose of the 2001 isolate developed conjunctivitis and seroconverted. I successfully isolated bacteria from each of these birds. To achieve this same high percentage of clinical disease and seroconversion with the 1995 isolate required a dose of 20,000 CCUs of this isolate. When the ID_{50} was calculated by the Reed Muench method, the virulence varied by a factor of 1.8 but was not significant (t= .032, d.f=1, p=.490). There were no differences between populations with regards to the dose necessary to establish infection.

I found no significant differences in eye score between the three populations (n= 107, R^2= .18, p = .08), but there was a significant difference in the severity of disease at week one between the two isolates (Figure 1; p< .0001). Seventy-eight percent of finches infected with the 1995 isolate were assigned a score of three or less at week one, while only 44% of finches infected with the 2001 isolate were assigned a score of three or less.

To assess differences in severity of infection, I compared the highest conjunctival scores assigned to each infected house finch. While there were no significant differences between the two isolates (n= 107, R^2= .09, p = .91) there was a significant difference between the three populations (Figure 2; p =.003). Post-hock analysis comparing the three populations revealed a significant difference between Alabama and Hawaii (p =.004), but no significant differences between Alabama and California (p = .096), or California and Hawaii (p = .112). Most finches in all three populations reached a maximum conjunctivitis score of 6, but 50% of the finches from Hawaii had a maximum score above that while only 32% of the finches from California, and 13% of the finches from Alabama had maximum conjunctivitis scores above 6. Thirty-three finches from all three populations and both isolates were necropsied at the end of the study. The lungs and trachea were examined for lesions indicating severe disease. There were no significant differences between populations (n= 33, R^2= .05, p = .606; n= 33, R^2= .18, p = .998) or between isolates (p = .437, p = .293) with regards to lesions detected in the lungs and tracheas respectively.

There was a significant difference between both the three populations of finches (n= 96, R^2= .15, p = .034), and the two isolates (p = .003) with regards to recovery from infection (Figure 3). Seventy-nine finches infected with the 1995 isolate recovered from

infection while only 50% of finches infected with the 2001 isolate recovered. Post-hock analysis revealed significant differences between recovery of Alabama and California finches ($p = .043$), and between California and Hawaii finches ($p = .029$), but no significant difference in recovery between Alabama and Hawaii finches ($p = .84$). Seventy-five and 77% of Alabama and Hawaii finches respectively recovered from infection with MG while only 50% of the California finches recovered by the end of the 12-week study. Eight house finches died while infected with MG, but there were no significant differences between population ($n= 96$, $R^2= .16$, $p = .319$), or isolate ($p = .997$) with regards to mortality.

DISCUSSION

Within the first few years following the appearance of MG in the eastern North American population of house finches there was an observed decrease in the prevalence of mycoplasmosis in the population. This study was designed to test for changes in the susceptibility of the host and the virulence of the pathogen following the epornitic. I predicted that if the decrease in disease prevalence among eastern house finches was due to natural selection in the bacteria, then the 2001 isolate from late in the epornitic would be less virulent than the 1995 isolate of MG, collected during the height of the eporntic. However, of the four measures of virulence that I tested three (Initial infective dose, severity of disease one week after inoculation, and rate of recovery), suggested that the 2001 isolate was more virulent than the 1995 isolate. Only one measure, overall severity of disease, was similar for both isolates.

These results suggest that the decline in prevalence of MG observed in wild house finches may not be due to decreased virulence in the MG. One explanation for the increased virulence observed in the 2001 isolate may be that virulence is increasing as the MG adapts to colonization and transmission in house finches. Mycoplasmas are obligate parasites that require firm attachment to host epithelial cells to sequester nutrients necessary for growth and reproduction from the host cell (Razin et al., 1998). Isolates collected from house finches shortly after invasion by MG should have been less adapted to attachment and reproduction on house finch epithelial cells, and such maladaptation could explain the higher dose of 1995 isolate needed to establish infection in the finches. Also, if the 2001 isolate is more proficient at attachment, it may sequester nutrients more efficiently and therefore have a faster rate of replication leading to an increase in the severity of disease observed one week after inoculation.

There were no significant differences between the two isolates in the severity of infection they caused, but a significantly higher percentage of house finches recovered from infection with the 1995 isolate than did finches infected with the 2001 isolate. The 2001 isolate caused a higher percentage of house finches to become chronically infected with moderate conjunctivitis, even 11 weeks after the onset of disease.

Unlike poultry infected with MG, house finches do not tend to develop a sneeze or cough during infection allowing the pathogen to become airborne. While exact modes of transmission have not yet been experimentally tested, house finches most likely transmit MG by direct contact with infected individuals or indirect contact through surface contamination. Infected house finches have ocular and sometimes nasal discharge and can be seen scratching at their irritated and swollen eyes or rubbing them on branches

and feeders. Chronic infection with moderate, irritating conjunctivitis should lead to higher rates of transmission. In fact, chronic MG infection is commonly observed in poultry where MG is very well adapted to its host (Ley and Yoder, 1997). The results of this study indicate that the house finch MG has become significantly more adapted to survival in its newly colonized host by causing chronic infections.

A less likely yet still plausible alternate explanation for the increased virulence observed in the 2001 isolate may be the number of laboratory passages. The 2001 isolate was only 3 passages removed from the original bird, but the 1995 isolate was eight passages removed. It is important to keep passage numbers as low as possible to avoid the loss of virulence genes that are not needed by the bacteria for survival in culture media, but passage numbers of both isolates were kept very low therefore there should not have been a loss of virulence factors such as attachment proteins.

I also predicted that if the decrease in prevalence observed in the wild was due to natural selection in the host then I would expect house finches from Alabama, who have been under selection for eight years, to better resist and recover from experimental infection than house finches from the two naive populations of California and Hawaii. I found no significant differences between any of the three populations of finches with regard to the dose needed to establish infection or the incubation period of either isolate. There was, however, a significant difference in the severity of the disease that house finches from the different populations developed. Finches from Alabama developed less severe disease than finches from either California or Hawaii. While a majority of house finches in all three populations developed a maximum eye score of 6, only 13% of

finches from Alabama developed a score of 7 or 8 while 32% of California finches had a maximum score of 7 or 8, and 50% of Hawaiian finches had an eye score of 7 or 8.

When I examined the percentage of house finches that recovered from infection with MG, 75% and 77% of Alabama and Hawaii finches respectively, recovered, while a significantly lower percentage (50%) of California finches recovered. While all three populations exhibited a range of responses to experimental infection with MG, in general, our results show that Alabama finches developed only a mild to moderate infection with a high rate of recovery, Hawaii finches developed a more severe infection, but also had a high rate of recovery, and California finches developed a moderate, more chronic infection. These results support, in part, my theory that house finches have evolved increased resistance to MG, but they do not fully explain the decreased prevalence observed in the wild especially when I account for the increased virulence of the bacteria. Future studies into transmission of MG from host to host, and a more detailed analysis of the molecular changes taking place within both host and parasite will provide amore complete understanding of the evolution of this host-parasite relationship and further explain the changes observed in the wild.

Table 1. Responses of wild birds from three locations experimentally inoculated with isolates of the house finch MG between September 2002 and August 2003. Results given as a number of positive birds (%).

		1995 Isolate			
Dose[a]	n	Clinical Disease	MG PCR Detection[b]	Antibody Detection[c]	Culture
2 CCUs	52	0	0	0	0
2 X 10^2 CCUs	50	2 (4)	0	11 (22)	0
2 X 10^4 CCUs	54	53 (98)	54 (100)	54 (100)	54 (100)
		2001 Isolate			
2 CCUs	54	0	6 (11)	16 (30)	0
2 X 10^2 CCUs	53	53(100)	53(100)	53(100)	53 (100)

[a] birds received 20uL SP4 containing a concentration of MG given in color changing units CCUs

[b] PCR performed on DNA extracted from choanal swabs

[c] antibodies detected using a serum plate agglutination assay

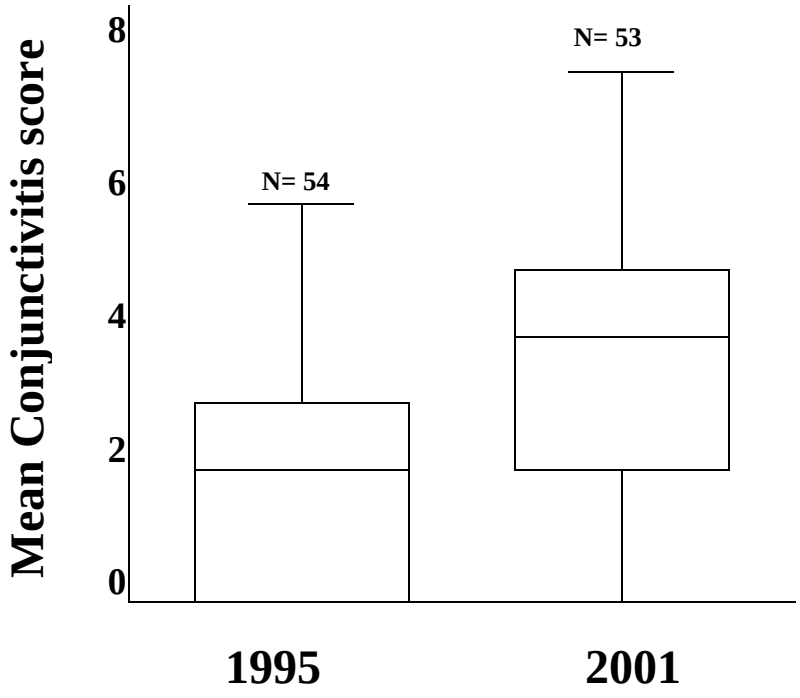

Figure1. Mean conjunctivitis score for house finches from all three populations one week after exposure to either the 1995 isolate or the 2001 isolate of *Mycoplasma gallisepticum* ($p < .0001$).

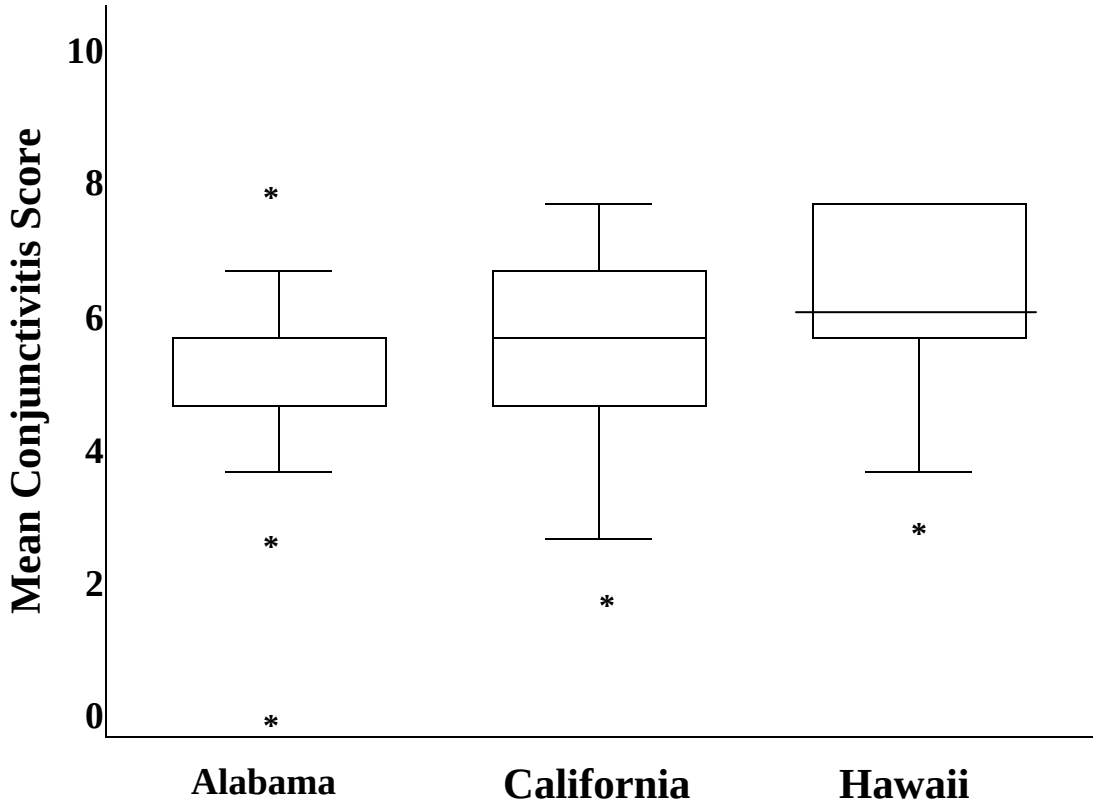

Figure 2. Mean of the highest conjunctivitis score given to house finches in the three populations exposed to the two isolates of *Mycoplasma gallisepticum*. Outliers are shown with an *. (p= .003).

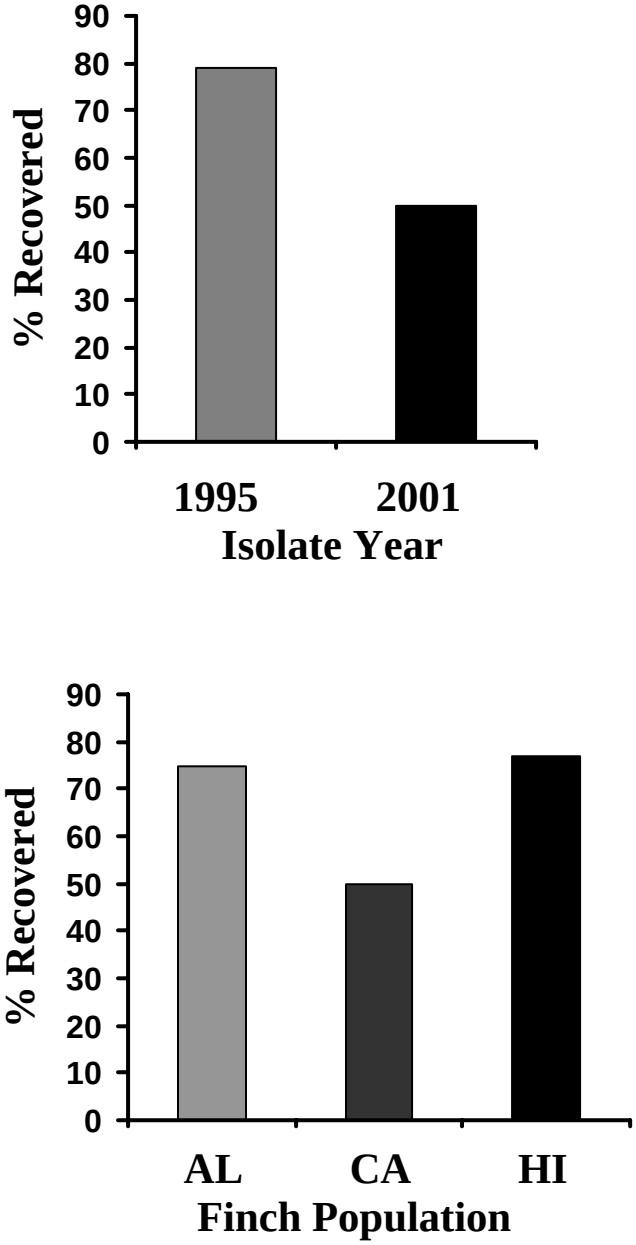

Figure 3. Percentage of house finches from three populations that recovered from infection with one of two isolates of Mycoplasma gallisepticum. ($p = .003$; $p = .034$).

V. SUSCEPTIBILITY OF WILD SONGBIRDS TO THE HOUSE FINCH STRAIN OF MYCOPLASMA GALLISEPTICUM

ABSTRACT: Conjunctivitis in house finches *(Carpodacus mexicanu*s), caused by *Mycoplasma gallisepticum* (MG), was first reported in 1994 and, since this time, has become endemic in house finch populations throughout eastern North America. Although the house finch is most commonly associated with MG-related conjunctivitis, MG has been reported from other wild bird species, and conjunctivitis (not confirmed as MG related) has been reported in over 30 species. To help define the host range of the house finch strain of MG and to better understand the effect of MG on other host species, I monitored a community of wild birds for exposure to MG and conducted experimental infections on nine avian species. For the field portion of our study, I conducted a 9-mo survey (August 2001 to April 2002) of wild avian species in a peri-urban environment on the campus of Auburn University. During this time 358 birds, representing 13 different families, were sampled. No clinical signs of mycoplasmosis were observed in any bird. Thirteen species from nine families had positive agglutination reactions for antibodies to MG, but all birds tested negative by polymerase chain reaction (PCR). Three mourning doves were PCR-positive for MG, but antibodies to MG were not detected. In the experimental infections, I exposed seven native avian species and two cage-bird species to MG (May 2000 to June 2002). After exposure, clinical disease was seen in all four

species from the family Fringillidae and in eastern tufted titmice *(Baeolophus bicolo*r). In addition, three other species were infected without clinical signs, suggesting that they may represent potential MG reservoirs.

Key Words: Baeolophus bicolor, Carduelis tristis, Carpodacus mexicanus, conjunctivitis, experimental infection, host range, *Mycoplasma gallisepticum,* passerine.

INTRODUCTION

Mycoplasma gallisepticum (MG) was originally isolated from a house finch *(Carpodacus mexicanu*s) with conjunctivitis in 1994. Within three years, this disease had spread through the entire eastern population of house finches in North America (Fischer et al., 1997; Dhondt et al., 1998). House finches are the most commonly reported passerine species with MG related conjunctivitis, but conjunctivitis has been reported from over 30 passerine species (Hartup et al., 2000, 2001; Mikaelian et al., 2001). MG infection has not been confirmed in most of these reported conjunctivitis cases, however. In field studies on wild birds, antibodies to MG have been detected by agglutination assays in over 20 wild bird species, including ten species in which conjunctivitis has been reported (Hartup et al., 2000; Luttrell et al., 2001). Although this suggests natural infection, the specificity of these serologic tests in wild bird species other than finches is unclear. MG has been confirmed by polymerase chain reaction (PCR) or culture in only six wild avian species: house finch, American goldfinch *(Carduelis tristi*s), purple finch *(Carpodacus purpureu*s), Eastern tufted titmouse *(Baeolophus bicolo*r), pine grosbeak *(Pinicola enucleato*r), and evening grosbeak *(Coccothraustes vespertinu*s)

(Fischer et al., 1997; Hartup et al., 2000; Luttrell et al., 2001; Mikaelian et al., 2001). Based on these limited data, the extent of infection within wild bird populations is unknown, and it is unknown whether wild avian species, other than house finches, are potentially affected by the house finch MG epidemic. Based on the list of species from which MG infection has been confirmed, we hypothesize that con-familial species would be more susceptible than more distantly related species.

To estimate prevalence of MG in a local population of songbirds and to help assess the specificity of the agglutination assay, I conducted a 9mo survey of wild avian species in a peri-urban environment on the Auburn University campus. To help define the host range of the house finch strain of MG and to better understand the effect of MG on potential target species, I conducted experimental infections on nine avian species.

MATERIALS AND METHODS

Field study. From August 2001 to April 2002, birds were captured in mist nets placed on agricultural research lands immediately adjacent to Auburn University, Lee County, Alabama (328359N, 858289W) under Federal Banding Permit No. 21661. The area consists of open mowed fields with brushy borders. Each bird was examined for clinical disease and was banded with a US Fish and Wildlife Service identification band. Blood (100 μl) was collected from the brachial vein and a micro tip swab was used to sample the choanal cleft (Roberts et al., 2001; Becton Dickinson and Co., Sparks, Maryland, USA).

Serum samples were tested for antibodies to MG by using a commercial serum plate agglutination (SPA) assay (Luttrell et al., 1996; Intervet Inc., Millsboro, Delaware).

In brief, the extent of agglutination was scored after 2 min on a scale from 0 to 4, with a score of 2 considered positive. We performed PCR using MG-specific primers and DNA extracted from choanal swabs (Roberts et al., 2001). Culture was attempted on 206 birds. For culture, swabs taken from the choanal cleft were placed into 3 ml of SP4 broth preheated to 37 C. After gentle vortexing, the swab was removed and the inoculated SP4 broth (Whitcomb, 1983) was incubated at 37 C for 5 weeks or until a color change indicated growth. Isolates were identified by PCR.

Species selection criteria for experimental infections. In our experimental infections I exposed seven avian species that are native to eastern North America and two cage-bird species to the house finch strain of MG. Four species are in the family Fringillidae: house finch, American goldfinch, pine siskin *(Carduelis pinus*), and purple finch. Natural MG infections have been confirmed in both American goldfinch and purple finch. Conjunctivitis has been reported in pine siskin but MG has not been isolated from this species (Hartup et al., 2000).

The tufted titmouse was selected based on previously reported sero-positive and PCR-positive results from this species (Luttrell et al., 2001). This is the only wild passerine species outside the family Fringillidae to test positive for MG by PCR (Luttrell et al., 2001). House sparrow *(Passer domesticus*: Passeridae) was included based on previous isolation of MG from this species in India (Jain et al., 1971). Antibodies to MG have been detected in house sparrows in the eastern United States (Stallknecht et al., 1982), and they have been experimentally infected with the R strain of MG, which was isolated from the trachea of infected birds for ten days after exposure. Clinical disease or lesions, however, was not reported (Kleven and Fletcher, 1983). Chipping sparrow

*(Spizella passerin*a: Emberizidae) was included because conjunctivitis has been observed in this species (Hartup et al., 2001), and sero-positive birds have been reported (Luttrell et al., 2001).

To expand the number of bird families tested without infecting additional wild bird species, I also included two cage-bird species. Zebra finches *(Taeniopygia guttat*a) are Australian passerines in the family Estrillidae. As a non-passerine test species I also included budgerigars *(Melopsittacus undulatu*s; order Psittaciaformes, family Psittacidae). These two bird species are easily maintained in captivity and are readily available; budgerigars are susceptible to infection with the R and P strains of MG (Bozeman et al., 1984; Brown and Butcher, 1991).

Experimental infections with MG. House finches *(n* =5), American goldfinches *(n* =13), eastern tufted titmice *(n* = 4), house sparrows *(n* = 9), pine siskins *(n* = 9), chipping sparrows *(n* = 10), and a purple finch *(n* = 1) were captured between May 2000 and July 2002 in Lee County, Alabama, using wire-mesh basket traps and mist nets under a federal collecting permit (MB784373–4) and a permit from the Alabama Department of Conservation (Montgomery, Alabama; No. 12). I also purchased ten budgerigars and nine zebra finches at local pet stores. All procedures involving these birds were approved by the Auburn University Internal Animal Care and Use Committee (0304-R-2271). Each bird was marked with an individually colored leg band. Each species was housed in an individual indoor, temperature-controlled room (1.6 m x 2.3 m x 2.6 m h) with natural light. Except for tufted titmice and the purple finch, all birds were housed as a free flying flock within the room. The purple finch and the tufted titmice were kept in individual wire cages (0.5 m^3); the tufted titmice cages were covered in silk foliage to simulate a

more wooded surrounding. Birds were fed millet and sunflower seeds and provided water ad libitum. Diets of chipping sparrows were supplemented with grass seeds; diets of house sparrows and tufted titmice were supplemented with mealworms. After a 1-wk acclimation period, 100 µl of blood and a choanal swab were collected from each bird. All birds were tested and confirmed to be PCR-negative and sero-negative prior to inoculation. Positive serologic results prior to infection were limited to the four budgerigars, but nonspecific agglutination with the SPA has been reported previously for this species (Bozeman et al., 1984).

After a minimum of 1 mo quarantine, birds were inoculated by placing 10 µl of SP4 media containing 1 X 10^7 color changing units (CCU)/ ml of MG into each eye. This dose is known to be infective in house finches (Farmer et al., 2002). The MG isolate used on all birds, except the purple finch, was obtained from a house finch from Auburn, Alabama, in 1999 and was two passages from the original isolation. The isolate of MG used to infect the purple finch was obtained from a house finch in Auburn, Alabama, in 2001 and was one passage away from the original culture. After inoculation, birds were monitored daily for the onset of clinical disease. Blood and swabs were collected every 7 to 10 days, for 10 weeks. The severity of conjunctivitis was scored for each eye on a scale of 0 to 3 as described by Roberts et al. (2001) with the addition of a fifth degree (an eye score of 4) in which the bird was completely blind due to conjunctival swelling. At 3 wk postinoculation (PI), MG culture was attempted as previously described. No birds were euthanized during the study, but dead birds collected in good condition were submitted to the Charles S. Roberts Alabama State Veterinary Diagnostic Laboratory (Auburn, Alabama) for necropsy. Submitted birds were examined for gross lesions.

Smears of the proventricular mucosa were collected, and Gram-stained, brain sections were collected for West Nile Virus testing by rt-PCR, and sections of intestine were collected for histology and bacteriology.

Serology was performed within 48 hr of collection and samples were tested blind with regard to the birds' clinical disease scores and their previous SPA scores. Averages and standard deviations were performed using Microsoft Excel 2000.

RESULTS

Field study. During the 9-mo field survey, 358 birds, representing 26 species in 13 families, were sampled. These included 53 recaptures. Clinical signs of conjunctivitis were not observed, and only three birds, all mourning doves, tested positive for MG by PCR. Forty-two birds (13.7%), representing 13 species from nine families, were SPA-positive (Table 1). Antibodies were detected in all three species representing Mimidae and in both species in Paridae. Sero-positive results were detected in three species that were included in the experimental trial (tufted titmouse, chipping sparrow, and house sparrow) (Table 1). All three PCR-positive mourning doves were sero-negative. With regard to the recaptured birds, six (7%) sero-positive birds were captured more than once during the study. Three of the birds, a tufted titmouse, a Northern mockingbird *(Mimus polyglotto*s), and a Northern cardinal *(Cardinalis cardinali*s) tested SPA-positive each time they were captured; however, four Northern cardinals changed in their serologic status from SPA-negative to SPA-positive or vice versa over a recapture range from 28 to 152 days. MG was not isolated from any of the 206 culture attempts, including the three PCR-positive mourning doves.

Experimental infections with MG. After inoculation, all finches (house finch, American goldfinch, pine siskin, and purple finch) and two of four tufted titmice developed conjunctivitis (Table 2). With the exception of one American goldfinch, all birds that developed signs of conjunctivitis did so within 1 wk PI. The American goldfinch that was normal at 1 wk PI developed conjunctivitis at 10 days PI (Table 3). Only a mild to moderate ocular discharge was observed in seven of nine pine siskins; conjunctivitis was not observed. Variation in the duration and severity of disease, both within and between the species, was observed. Pine siskins exhibited a mild and short-lived clinical disease lasting less than 3 wk (Table 3); this was mirrored by a short-lived antibody response (Fig. 1). The purple finch developed severe conjunctivitis that resolved within 4 wk, although MG (as detected by PCR) and antibodies to MG could still be detected for 10wk (Fig. 1). Only two tufted titmice developed severe conjunctivitis, but all four developed antibodies that could be detected 8 to 10 wk PI; MG was detectable by PCR in these birds an average of 4 wk after exposure (Fig. 1). The house finches and American goldfinches developed moderate to severe conjunctivitis that took an average of 7 wk to resolve (Fig. 1); four American goldfinches and one house finch remained clinically ill throughout the 10-wk study (Table 3). MG was detected for an average of 4 wk in the American goldfinches but could be detected in the house finches until clinical disease was resolved. House finches had a longer lasting antibody response than did the American goldfinches (Fig. 1). MG was successfully reisolated from house finches, American goldfinches, and one tufted titmouse (Table 2).

House sparrows, chipping sparrows, zebra finches, and budgerigars did not develop clinical disease. Antibodies to MG, detected in three zebra finches, did not

persist beyond the first week. Antibodies were detected from house sparrows for as long as 5 wk PI. Antibodies to MG could not be detected in chipping sparrows or budgerigars because of the ambiguity of the serologic test. MG was detected by PCR in the choanal cleft of house sparrows, zebra finches, and budgerigars, but not in the chipping sparrows (Table 2). MG was detected in three zebra finches, two budgerigars, and two house sparrows for 3 wk after exposure.

For seven of the nine species, birds developed a clear antibody response, but the duration of detectable antibodies was variable and most birds tested negative at the conclusion of the study. For both budgerigars and chipping sparrows, individual birds shifted between high agglutination and low agglutination reactions with no consistent pattern or connection to other parameters of disease. Based on these results, SPA results were regarded as inconclusive.

Two American goldfinches and one tufted titmouse died while infected with MG (Table 2). Five house sparrows and three pine siskins died but were not infected with MG. Three pine siskins, four house sparrows, and one tufted titmouse were submitted for necropsy. The Eastern tufted titmouse was positive for West Nile Virus and the lungs had acute multifocal pneumonia. The pine siskins were all positive for megabacteriosis in the proventriculus, and the house sparrows were diagnosed with coccidiosis.

DISCUSSION

This is the first report of mourning doves, or any bird in the order Columbiformes, testing positive for MG by PCR. Although three mourning doves from two capture dates were positive by PCR, the lack of antibodies to MG or culture success suggests that these

birds were not currently infected with MG. It is possible that these birds were sampled in the very early stages of infection prior to antibody development, but this possibility is not consistent with negative culture results. Alternately, they may have been infected with a related species of mycoplasma. Although mourning doves were the only PCR-positive species in our field survey, I had 13 SPA-positive species, ten of which had previously been reported with conjunctivitis (Hartup et al., 2001) and seven of which had previously been reported as sero-positive for MG (Hartup et. al., 2000; Luttrell et al., 2001). For this same period 21% of house finches caught in the area were sero-positive for MG (unpubl. data).

Experimental infections demonstrate that nonspecific agglutination can occur in wild birds. Ambiguous serologic findings have been reported for poultry (Glisson et al., 1984), house sparrows (Kleven and Fletcher, 1983), and budgerigars (Bozeman et al., 1984), but little is known about the validity of the test in wild avian species other than house finches. During the experimental infections, the SPA was an accurate and inexpensive tool to monitor antibodies in seven of nine species but was unreliable in budgerigars and chipping sparrows. Chipping sparrows were also one of the 13 SPA-positive wild bird species detected during the field survey and it is possible that false positive results were present in other species. The SPA is a useful tool for screening a large number of birds for antibodies to MG in species for which the validity of the test has been demonstrated or in which infection has been documented. With other species, positive results should be viewed with caution.

All species in the family Fringillidae were susceptible to experimental infection with MG. They developed clinical disease and sero-converted, and MG was detectable by

PCR for several weeks PI. Tufted titmice were the only species outside of Fringillidae to develop clinical disease associated with MG infection. House sparrows, zebra finches, and budgerigars were infected without clinical disease. Chipping sparrows were the only species in which infection could not be confirmed by PCR.

One of the most intriguing results in this study is the high susceptibility of American goldfinches to infection with MG. Although house finches and American goldfinches aggregate at feeders and can often be seen feeding side by side, American goldfinches are less frequently observed with conjunctivitis (2%, Hartup et al., 2001; 3%, Luttrell et al., 2001) than are house finches (20%, Dhondt et al., 1998; 27%, Luttrell et al., 2001). The difference in infection rates of house finches and American goldfinches in the wild may be due to many factors, such as lack of transmission of MG between house finches and American goldfinches, inefficient transmission between goldfinches, or variation in susceptibility of the two species. I demonstrated here that American goldfinches are as susceptible as house finches to experimental infection. Species related differences in the number of reported cases of conjunctivitis are not understood with these species and others and will require further research to identify specific risk factors that may be associated with both MG susceptibility and the potential for transmission.

Both pine siskins and the purple finch were susceptible to infection yet neither species was as severely affected as house finches or American goldfinches. Wild pine siskins have been observed with conjunctivitis (Hartup et al., 2001), although none of the birds tested have been sero-positive (Hartup et al., 2000; Luttrell et al., 2001). One previously captured purple finch with conjunctivitis tested positive by PCR and culture; and it, along with two others, tested sero-positive (Hartup et al., 2000). This study

identifies pine siskins as a potential MG carrier. Interpretation of results from the purple finch is difficult based on sample size. The bird did not become as severely infected as the goldfinches and house finches, but this may be because of individual variation and not representative of the purple finch population. Based on results from this single bird, however, purple finches can remain infected for up to 4 wk. I also demonstrate that the SPA test is valid for both of these species.

It has been suggested that tufted titmice may be carriers of MG. Antibodies to MG have been reported from this species (Hartup et al., 2000; Luttrell et al., 2001), but before this study, this was the only species outside of the family Fringillidae from which MG had been detected by PCR but not culture (Luttrell et al., 2001). In the few reports of tufted titmice with conjunctivitis, infection with MG also was not con-firmed (Hartup et al., 2001). During the field survey we captured nine tufted titmice, four of which were SPA-positive. One of these four birds was recaptured twice and remained SPA-positive. In the experimental infection of four tufted titmice, they were the only nonfringillid species that developed conjunctivitis in response to infection. This species appears to be less likely to develop clinical disease than house finches or American goldfinches given that only half of the individuals developed conjunctivitis at the same dose that produced disease in 100% of house finches and American goldfinches. A larger number of tufted titmice would be needed to more accurately determine the percentage of individuals that develop clinical disease. However, their highly nervous behavior makes them very difficult to keep in captivity for extended periods.

House sparrows with conjunctivitis have been reported in the wild (Hartup et al., 1998), but my data show that, although MG could be detected by PCR in exposed house

sparrows for up to 3 wk PI, they never developed clinical disease. MG has been isolated from both wild and experimentally infected house sparrows (Jain et al., 1971; Kleven and Fletcher, 1983), and in the field survey, six of 33 house sparrows were SPA-positive; none of these birds had clinical disease. If house sparrows function as MG carriers, they are most likely nonclinical carriers.

Budgerigars have been experimentally infected with both the R strain and MG (P) strain isolated from yellow-naped Amazon parrots *(Amazona auropalliat*a), and these strains were detected in the trachea 35 and 21 days PI, respectively (Bozeman et al., 1984). In a second study, budgerigars infected with the R strain developed severe clinical signs at 7 days PI; clinical signs were present to 21 days PI (Brown and Butcher, 1991). Clinical signs were not observed in budgerigars infected with the house finch strain of MG in this study, but I was able to detect MG by PCR for 3 wk PI. This suggests that budgerigars are less susceptible to the house finch strain of MG than either the psittacine (P) or poultry (R) strains. As with the budgerigars, zebra finches did not develop clinical signs, but MG was detected in infected birds for up to 3 wk PI. Unfortunately, I was unable to determine whether chipping sparrows or budgerigars developed antibodies in response to these experimental infections. I was able to detect antibodies in the zebra finches, but only in a few birds and only in the first week PI.

This is the first experimental study in which species other than house finches were challenged with the house finch strain of MG. Although this MG strain infected a wide range of hosts, including a species of psittacine, disease was only confirmed in species in Fringillidae and Paridae. All species representing Fringillidae and the tufted titmice, which represented the only species from Paridae, developed clinical disease. These

results are consistent with previous confirmed reports of mycoplasmal conjunctivitis in wild birds, which are currently restricted to house finch, American goldfinch, and evening and pine grosbeak. Blue jays *(Cyanocitta cristat*a) (Ley et al., 1996) and tufted titmice can develop conjunctivitis in captivity when exposed to the house finch strain of MG, but naturally acquired disease has not been confirmed in these species. Results from both the field and experimental work indicate that several bird species can be infected with MG without demonstrating clinical disease. This suggests that other species may be involved as reservoirs for this pathogen. Further work to document and understand transmission within such potential reservoirs is critical to our understanding of the emergence and epidemiology of this disease.

Table 1. Species and number of wild birds captured in Auburn, Alabama between August 2001 and April 2002, and tested *Mycoplasma gallisepticum* by serum plate agglutination. Test results are given as number of positive samples/ total number sampled.

Family	Species	Common Name	Serology[a]
Columbidae	*Streptopelia decaocto*	Eurasian collared dove	0/1
Columbidae	*Zenaida macroura*	Mourning dove	3/54 (2)[b]
Corvidae	*Cyanocitta cristata*	Blue jay	1/3
Paridae	*Poecile carolinensis*	Carolina chickadee	2/17 (9)
Paridae	*Baeolophus bicolor*	Tufted titmouse	4/17 (8)
Troglodytidae	*Thryothorus ludovicianus*	Carolina wren	0/6 (2)
Troglodytidae	*Troglodytes aedon*	House wren	0/1
Regulidae	*Regulus satrapa*	Golden-crowned kinglet	0/5 (1)
Regulidae	*Regulus calendula*	Ruby-crowned kinglet	0/9 (2)
Turdidae	*Catharus guttatus*	Hermit thrush	0/3 (1)
Turdidae	*Turdus migratorius*	American robin	0/2
Mimidae	*Dumetella carolinensis*	Gray catbird	2/2
Mimidae	*Mimus polyglottos*	Northern mockingbird	3/11 (5)
Mimidae	*Toxostoma rufum*	Brown thrasher	4/9 (3)
Parulidae	*Dendroica coronata*	Myrtle warbler	0/27
Parulidae	*Icteria virens*	Yellow-breasted chat	0/2
Emberizidae	*Pipilo erythrophthalmus*	Eastern towhee	0/7 (2)
Emberizidae	*Spizella passerina*	Chipping sparrow	4/20
Emberizidae	*Melospiza melodia*	Song sparrow	0/3
Emberizidae	*Melospiza georgiana*	Swamp sparrow	0/1
Emberizidae	*Zonotrichia albicollis*	White-throated sparrow	0/27 (4)
Cardinalidae	*Cardinalis cardinalis*	Northern cardinal	5/49 (14)
Icteridae	*Agelaius phoeniceus*	Red-wing blackbird	1/1
Icteridae	*Molothrus ater*	Brown-headed cowbird	1/7
Fringillidae	*Carduelis tristis*	American goldfinch	6/41
Passeridae	*Passer domesticus*	House sparrow	6/33

[a]Total number of birds with a score of ≥ 2 on rapid agglutination test

[b]Number of birds recaptured

Table 2. Responses of wild birds experimentally inoculated with the house finch strain of MG (# positive individuals/ # tested). Birds were sampled for the presence of MG by PCR or serology every 7 to 10 days for 10 weeks between May 2000 and July 2002.

Species	Clinical Disease	MG PCR detection	Serlogy[a]	MG culture[b]	Mortality
House finch	5/5	5/5	5/5	5/5	0/5
American goldfinch	13/13	13/13	13/13	6/13	2/13
Pine siskin	7/9	9/9	9/9	0/9	3/9
Purple finch	1/1	1/1	1/1	0/1	0/1
Tufted titmouse	2/4	4/4	4/4	1/4	1/4
Chipping sparrow	0/10	0/10	ND[c]	0/10	0/10
House sparrow	0/9	8/9	8/9	0/9	5/9
Zebra finch	0/9	7/9	3/9	0/9	0/9
Budgerigar	0/10	5/10	ND	0/10	0/10

[a]antibodies detected by serum plate agglutination

[b]birds were cultured at 3 weeks p.I.

[c]No data was obtained on these species due to ambiguous results

Table 3. The progression of clinical disease (# positivel/ # tested) over ten weeks in wild birds inoculated with a house finch strain of *Mycoplasma gallisepticum.*

	Weeks postinoculation						
Species	1	2	3	4	6	8	10
House finch	5/5[a]	5/5	5/5	5/5	4/5	3/5	1/5
American goldfinch	12/13	13/13	11/12	9/12	12/12	7/11	4/11
Pine siskin	7/9	5/9	0/7	0/6	0/6	ND[b]	0/6
Purple finch	1/1	1/1	1/1	1/1	0/1	0/1	0/1
Tufted titmouse	2/4	2/4	2/4	2/4	1/3	0/3	0/3
Chipping sparrow	0/10	0/10	0/10	0/10	0/10	0/10	0/10
House sparrow	0/9	0/9	0/9	0/8	0/8	0/5	0/4
Zebra finch	0/9	0/9	0/9	0/9	0/9	0/9	0/9
Budgerigar	0/10	0/10	0/10	0/10	0/10	0/10	0/10

[a]positive birds scored one or higher in one or both eyes

[b]No data were collected for this species at eight weeks postchallenge

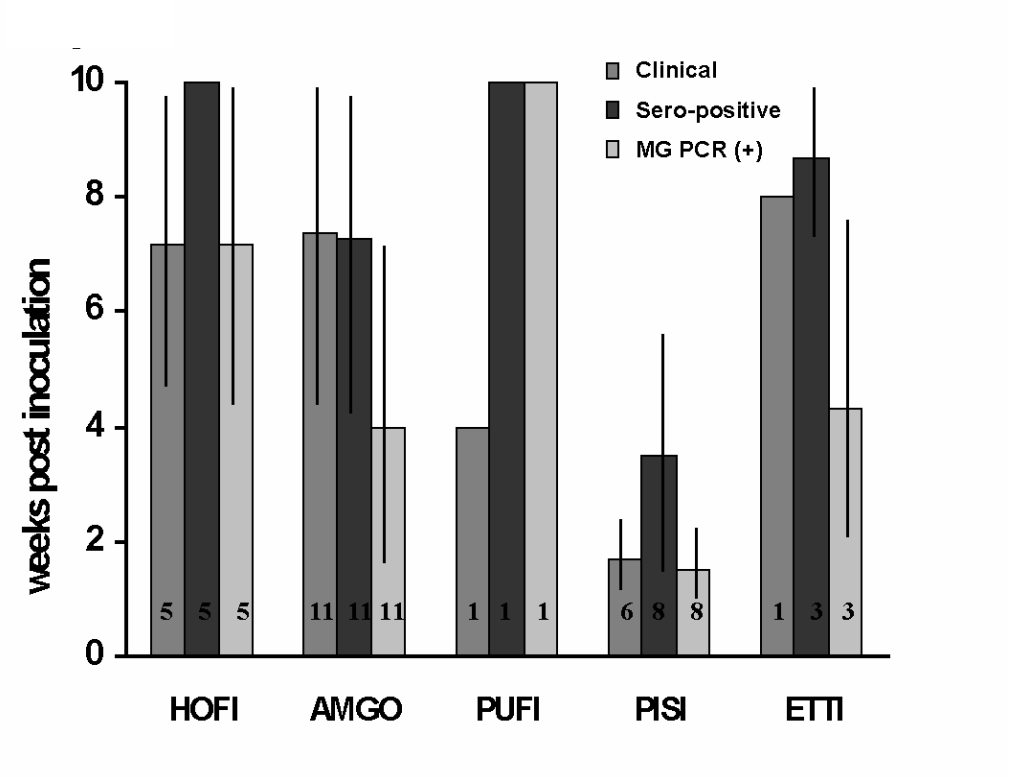

Figure1. Average duration of clinical disease, detectable antibodies, and positive PCR results from species exhibiting clinical disease in response to experimental inoculation with the house finch strain of MG. Bars are SD, numbers are sample size. HOFI = house finch, AMGO = American goldfinch, PUFI = purple finch, PISI = pine siskin, and ETTI = Eastern tufted titmouse.

VI. CONCLUSION

The evolutionary dynamics of host-parasite relationships, especially how and why virulence is maintained, is of interest to many scientists and has applications in fields from vaccine development, agriculture and conservation biology. Though details about many specific host-parasite systems are available, our understanding of the evolution of parasite virulence and host resistance is limited. In this study I used the MG-house finch system to test current ideas about the evolution of host-parasite relationships.

The traditional view on virulence evolution held that all pathogens should evolve to a state of benign existence in their hosts because destruction of their host would be detrimental to long-term pathogen survival (Smith, 1934; Zinsser, 1935; Dubos, 1965; Hoeprich, 1987). With the house finch-MG system the decline in prevalence seen in the wild would seem to suggest that virulence of the house finch MG strain may indeed have decreased. I predicted that if the decrease in the prevalence of disease was due mainly to changes in the bacteria, the isolate collected during the height of the epidemic should exhibit a higher virulence than the isolate collected later. However, in three of the four parameters of virulence that I tested (Initial infective dose, severity of clinical disease one week post-exposure, and rate of recovery), the later isolate was more virulent than the earlier isolate.

The increased virulence I observed may be more readily explained by the adaptive model of virulence evolution in which virulence is an unavoidable consequence of parasite replication through the use of host tissues, metabolic waste, and/or toxins (Levin, 1996; Roode et al., 2005). As the MG became better adapted to its new host and increased its rate of replication therefore increasing the number of transmissible bacteria, the damage caused to the house finch also increased.

My results also suggest that later strains of MG are more likely to cause chronic infection, often accompanied by conjunctivitis. While exact modes of transmission have not yet been experimentally tested, house finches most likely transmit MG by direct contact with infected individuals or indirect contact through surface contamination. Infected house finches have ocular and sometimes nasal discharge and can be seen scratching their irritated and swollen eyes or rubbing them on branches and feeders. Chronic, mild conjunctivitis in a mobile bird should lead to higher rates of transmission. In fact, chronic infection, with or without overt signs of disease, is the hallmark of pathogenic mycoplasmas (Maniloff, 1992). Overall, the results of my study suggest that the house finch MG strain is becoming better adapted to survival in its newly colonized host.

At the same time a pathogen is adapting to its new host, the host is undergoing selection for resistance through eradication of highly susceptible hosts. I predicted that if the decrease in prevalence observed in the wild was due to natural selection in the host then I would expect house finches from the Alabama population that has been under selection for eight years to better resist and recover from experimental infection than house finches from the two naive populations of California and Hawaii. While all three

populations exhibited a range of responses to experimental infection with MG, in general, my results demonstrated that Alabama finches develop milder infection with a higher rate of recovery, Hawaii finches develop a more severe infection, but also had a high rate of recovery, and California finches develop a moderate, more chronic infection. These results support, in part, my prediction that the eastern house finch population is more resistant to MG, but they do not fully explain the decreased prevalence observed in the wild. Transmission plays a crucial role in any host-parasite relationship and my study was not designed to test any aspect of transmission. Changes in the rates of transmission may also explain the overall decrease in the prevalence of MG in the eastern range of the house finch and need to be tested.

The origin of the house finch MG strain is unknown. Early in the epidemic it was assumed that a strain of poultry MG, most likely from back-yard chickens that live in frequent association with wild birds, expanded its host-range and began infecting house finches (I have this reference, I just have to find it). Alternately, it may have arisen by mutation from another MG source, perhaps present in wild birds. By whatever means, the newly emerged house finch MG strain does infect and cause disease in other wild bird species (Ley et al., 1996; Ley et al., 1997; Mikaelian et al., 2001), but the susceptibility of other wild birds has not been previously tested.

Through experimental infection I found that MG caused clinical disease in all Fringillid species tested and in one non-Fringillid species. Perhaps more importantly to the natural history of this MG strain, two other non-Fringillid species could be infected without the development of overt disease. These results suggest that other songbird species may serve as reservoirs for MG and may also play an important role in

transmission of disease. One of the most intriguing results of the infection in songbirds is the high susceptibility of Fringillid species, especially American goldfinches, to experimental infection with MG. Although house finches and American goldfinches aggregate at feeders and are often seen feeding side by side, there was no concurrent epidemic in the goldfinch nor in any other related species. It is possible that the house finch MG originated from a passerine species in which it caused little or no disease, and so has remained undetected until now. Prior to the epidemic, very few studies examined the presence of MG in wild passerines (Jain et al., 1971; Shimizu et al., 1979; Stallknecht et al., 1982), but evidence of MG infection was only detected in two sparrow species, one in Japan, and the other in India. Additional phylogenetic analysis of isolates collected from various wild passerine species as well as from domestic poultry may help determine the true origin of the house finch MG.

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