ANAPLASMA PHAGOCYTOPHILUM INFECTION IN TWO SPECIES OF PASSERINE BIRD, AMERICAN ROBINS AND GRAY CATBIRDS: AN ASSESSMENT OF RESERVOIR COMPETENCE AND DISEASE

By

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Fisheries and Wildlife

2011

ABSTRACT

ANAPLASMA PHAGOCYTOPHILUM INFECTION IN TWO SPECIES OF PASSERINE BIRD, AMERICAN ROBINS AND GRAY CATBIRDS: AN ASSESSMENT OF RESERVOIR COMEPTENCE AND DISEASE

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Anaplasma phagocytophilum (Ap) is the agent of human granulocytic anaplasmosis, an emerging infectious disease and a common tick-borne disease in the US and Europe. Ap is transmitted by the blacklegged tick (*Ixodes scapularis),* and small and medium-sized mammals are the typical reservoir species. Birds are also exposed to Ap and it has been hypothesized that birds aid in the dispersal of the pathogen and vector during migration. We exposed wild-caught gray catbirds (*Dumetella carolinensis)* and American robins (*Turdus migratorius;* n=10/species) to Ap-infected *I. scapularis* nymphs (day 0) and observed changes to temperature and mass during the exposure period. Four BALB/c mice (*Mus musculus)* served as controls throughout the experiment. Uninfected larvae were attached to each bird and mouse on days 7, 14, 42 and 77 to assess transmission rate and duration, blood samples were taken on days 3, 5, 7, 9, 14 for bacteremia and days 14 and 28 for serology. None of the birds were found to be bacteremic, transmit at appreciable levels, nor develop Ap-specific antibodies. All mice, however, were bacteremic on day 7 and transmitted through day 42 and developed Ap-specific antibodies. Exposed catbirds may have developed a fever due to exposure, though no other signs of disease were detected. Our results show that catbirds and robins are unlikely to play a significant role in the maintenance and transmission of Ap.

ACKNOWLEDGEMENTS

This work was supported by grants from Michigan State University College of Veterinary Medicine Endowed Research Fund, the Department of Fisheries and Wildlife, the Ecology, Evolutionary Biology and Behavior Program, Sigma Xi Grants-in-Aid of Research, American Ornithologists Union Research Grant, and the Hal and Jean Glassen Memorial Foundation. I am grateful for their support.

I am thankful to the Michigan Department of Natural Resources and the City of Lansing as well as Rich and Brenda Keith for their assistance and allowing me to conduct field research on their property. I appreciate the generosity of Jeff Landgraf and the Quantitative Genomics lab for sharing their time, knowledge, lab space and supplies and for keeping my quantitative polymerase chain reaction going day and night. Durland Fish provided the pathogen-free *Ixodes scapularis* larvae used in this project and I appreciate this generous gifts. Michael Levin's lab provided the infected and uninfected *Ixodes scapularis* nymphs and helpful guidance and advice throughout the experiments. Dan Ardia allowed us access to a thermometer and thermocouple, which was appreciated. Lisa Kaloustian, Nicole Grosjean and Steve Bolin and the Diagnostic Center for Population and Animal Health contributed time and resources to run the immunofloresence assays for me and I am thankful for their assistance.

I am very grateful to my colleagues in the Avian Health and Disease Ecology Lab, particularly my fellow graduate students Dustin Arsnoe and Tiffanie Hamilton for their help catching birds and collecting samples, and their support through all the challenges of this project. I was also fortunate enough to be absorbed by the Tsao Tick

Lab and provided guidance by my colleagues there, including Sarah Hamer, Isis Kuczaj, Jen Sidge and Genevieve Pang. There were many field and laboratory technicians that made this study possible, and I was very lucky to have all their assistance, especially Andrew Brown, Lydia Kramer, Nathan Spala, Steven Gray, Daniel Cook, Nicollette Purcell, Grace Hirzel, and Sarah Privett.

I received excellent guidance and advice from my committee chair and members: Jen Owen, Jean Tsao, Daniel Hayes, Ned Walker and Linda Mansfield who were always willing to share their time and wisdom. My friends and loved ones particularly Andy Flies, for his unbounded technical, mental and emotional support, Cory Brant, Lissy Goralnik, Kim Winslow, Stacie Auvenshine, and my family for their advice, assistance and encouragement; for all of them my love and gratitude grows daily.

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CHAPTER 1

THE ROLE OF BIRDS IN THE ECOLOGY AND EMERGENCE OF *ANAPLASMA PHAGOCYTOPHILUM:* A REVIEW.

INTRODUCTION

In 1992, a Wisconsin man fell ill with the first human case of human granulocytic anaplasmosis (HGA), a disease caused by *Anaplasma phagocytophilum* (Ap; (Chen, Dumler et al. 1994; Dumler, Choi et al. 2005). Since its discovery in the US, Ap has increased in incidence and distribution, making it the second most common tick-borne illness in the Midwestern and Northeastern US after Lyme disease (Dahlgren, Mandel et al. 2011; CDCP January 2011). This emergence is likely due to improved surveillance and detection techniques and the spread of the main vector of Ap, *Ixodes scapularis* (Ogden, Woldehiwet et al. 1998; Hamer, Tsao et al. 2010; Dahlgren, Mandel et al. 2011).

The main reservoirs for this pathogen are believed to be small and medium mammals (Levin, Nicholson et al. 2002), and until recently, birds were not thought to be competent reservoirs (Alekseev, Dubinina et al. 2001; Skotarczak, Rymaszewska et al. 2006). However, birds are known to be exposed to the pathogen (Daniels, Battaly et al. 2002; Ogden, Lindsay et al. 2008) and at least two species (American robins and veerys) have been implicated as potentially capable of transmitting the pathogen to uninfected larvae (Daniels, Battaly et al. 2002). Furthermore, migrating birds have been found to carry infected nymphs, suggesting that birds may play a role in the long-distance dispersal of the vector and pathogen (Ogden, Lindsay et al. 2008; Hildebrandt, Franke et al. 2010). However, for birds to play a role in the emergence of a pathogen, certain

physiological and ecological criteria must be met. This paper will review the history of Ap and the likelihood that birds play a role in its ecology, transmission and dispersal.

HISTORY

Ap was first described in Scotland in 1940 (Gordon, Brownlee et al. 1940) as the agent of tick-borne fever. Since that time, it has been found in European wild (roe deer, reindeer) and domestic (sheep, goats, cattle, horse, dogs, cats) animals and is recognized as the most wide-spread tick-borne infection of European animals (Strle 2004; Stuen 2007).

Though Ap was discovered in Europe, a similar agent was soon identified in America to cause granulocytic anaplasmosis in horses (Gribble 1969), dogs (Madewell and Gribble 1982), and humans (Chen, Dumler et al. 1994). After Ap was discovered as a human pathogen in the US, cases of HGA have been identified throughout Europe, though the European strains of Ap appear to be less virulent in humans than the American strains (Massung, Mather et al. 2006).

In 2001, a reorganization of the family *Anaplasmatacea* in the order *Rickettsiales* reclassified the three species formerly known as *Ehrlichia equi*, and *Ehrlichia phagocytophilum* (formerly *Cytoesetes phagocytophila* and *Rickettsia phagocytophila*) and the human granulocytic ehrlichiosis agent, as Ap, based on their molecular (99.1% homology), phenotypic and serologic similarities (Dumler, Barbet et al. 2001).

BACTERIA BIOLOGY

The bacteria in this species are small (0.2–1.0 μm in diameter), gram negative, obligate intracellular organisms. They replicate by binary fission in the early endosome of granulocytic white blood cells: neutrophils in mammals and heterophils in birds and reptiles (Foggie 1951; Chen, Dumler et al. 1994; Walker and Dumler 1996; Nieto, Foley et al. 2009). Although they are gram negative, they lack lipopolysaccharide polysynthetic machinery (Lin and Rikihisa 2003) and do not stain well with a Gram stain; the bundles of bacteria (called morulae) that develop in granulocytes are most visible after a Giemsa, Leishman or other differential staining of a blood smear (Foggie 1951).

Ap is unique among the rickettsia because it has a significantly higher number of functional pseudogenes which it uses to switch the expression of its major surface protein (MSP; (Foley, Nieto et al. 2009). The MSP (P44) region of the genome codes for an adhesion molecule that allows Ap to bind to the PSGL-1 ligand on neutrophils before infecting them (Park, Choi et al. 2003). These surface proteins are also the immunodominant antigen of Ap, thus by switching their expression, Ap can avoid detection by the immune system (Foley, Nieto et al. 2009). Furthermore, the hypervariablility of this and other regions that code for antigenic proteins allows for detailed phylogenetic analysis.

GRANULOCYTIC ANAPLASMOSIS

Transmission of Ap by infected tick bite has been shown to occur in 24-36 hours (Hodzic, Fish et al. 1998; Katavolos, Armstrong et al. 1998; des Vignes, Piesman et al. 2001) and symptoms typically emerge 5 to 21 days after tick bite (Novakova and Vichova 2010). The quantifiable signs of anaplasmosis (fever and leucopenia) were first

identified in sheep and termed tick-borne fever (Taylor, Holman et al. 1941). Since that time, many other domestic animals have been found to develop similar symptoms in response to Ap exposure.

Humans are considered an accidental host for Ap and infection, which alters white blood cell composition and function, can develop into HGA. Though Ap infections are mostly asymptomatic, it can result in symptoms such as fever, malaise (general discomfort), myalgia (muscle pain), headache, gastrointestinal problems (nausea, vomiting, diarrhea), skin rash, cough and damage to the liver (Bakken, Goellner et al. 1998; Dumler, Choi et al. 2005). Intensive care is required in 5-7% of HGA cases and death is a very rare outcome, mostly resulting from secondary infections caused by the weakened immune system (Bakken, Krueth et al. 1996; Dumler, Choi et al. 2005). The infection is usually cured with antibiotics, particularly doxycycline, rifampin, and levofloxacin (Maurin, Bakken et al. 2003). Patients typically develop detectable levels of IgM antibodies by 5-14 days post infection and IgG antibodies 10-28 days post-infection (Woldehiwet and Scott 1982; Novakova and Vichova 2010).

STRAIN DIVERSITY

Different Ap strains have been identified through variations in the 16S rRNA intergenic spacer region, *gro*ESL heat shock operon, *ank* and *msp2* and *msp4* genes. The 16S, *gro*ESL and *ank* housekeeping genes are conserved and thus informative for deep phylogenetic analyses while the more variable antigenic surface protein regions *msp* (p44) are informative for separating more closely related strains of Ap.

Based on analysis of these genes, studies have separated two clades in the US (Northeastern and Midwestern/Western), from a European clade (Massung, Owens et al. 2000; Morissette, Massung et al. 2009). Furthermore, there are two main strains of Ap (Ap-ha and Ap-variant 1) that vary by two base pairs at the16S rRNA region (Belongia, Reed et al. 1997; Massung, Slater et al. 1998; Massung, Mauel et al. 2002) yet have distinctly different ecologies. Ap-variant 1 has a strong ruminant tropism and shares an evolutionary history with European ruminant strains while Ap-ha infects humans, dogs, rodents and medium-sized mammals (De La Fuente, Massung et al. 2005; Portillo, Santos et al. 2005; Massung, Levin et al. 2007; Morissette, Massung et al. 2009). Both are found throughout the US.

There is a high degree of heterogeneity of *msp* regions in Ap-variant1 which allows for identification of more recently diverged strains (De La Fuente, Massung et al. 2005). For example, strains that infect donkey, horse, bison and sheep can be separated according to their host tropism using the hypervariable *msp* region (De La Fuente, Massung et al. 2005). In fact, this *msp* heterogeneity is likely be related to the host reservoir diversity displayed by Ap-variant 1 (De La Fuente, Massung et al. 2005).

Ap-ha and Ap-variant 1 overlap in range and though the relative prevalence of each strain varies by location, Ap variant-1 is more common where they do co-exist (Massung, Mauel et al. 2002; Courtney, Dryden et al. 2003).

Antibodies developed to Ap-variant 1 strains are not fully protective for heterologous nor homologous infections (Sun, Ijdo et al. 1997; Levin, Coble et al. 2004), possibly as a result of the hypervariable *msp* region to which a host develops antibodies. Repeat susceptibility has been found in goats (Massung, Mather et al. 2006), sheep

(Stuen, Artursson et al. 1998) and mice (Sun, Ijdo et al. 1997; Levin and Fish 2000; Levin, Coble et al. 2004), though intensity of infection appears to diminish with repeat infection (Levin and Fish 2000; Levin, Coble et al. 2004).

TRANSMSISSION ECOLOGY

Vectors

Throughout its range across North America, Europe and Asia, Ap is typically transmitted to humans via hard ticks in the *I. persulcatus* complex (Pancholi, Kolbert et al. 1995; Richter, Kimsey et al. 1996; Telford, Dawson et al. 1996; Cao, Zhao et al. 2000). In the US, the most common vector of Ap is *I. scapularis*, though in western regions, *I. pacificus* is the main vector (Pancholi, Kolbert et al. 1995; Telford, Dawson et al. 1996; Barlough, Madigan et al. 1997; Ogden, Woldehiwet et al. 1998). In Europe and Asia, *I. ricinus* and *I. persulcatus* ticks, respectively, dominate the transmission ecology (Cao, Zhao et al. 2000; Alekseev, Dubinina et al. 2001; Strle 2004).

The nidiculous tick, *I. trianguliceps* has also been found infected (Ogden, Bown et al. 1998) and may play a role in the enzootic transmission of Ap-ha among wood mice (*Apodemus sylvaticus*) and bank voles (*Clethrionomys glareolus*) in one system in England (Bown, Begon et al. 2003). *I. dentatus* maintains Ap-ha variants among rabbits in the US (Goethert and Telford 2003). *Haemophysalis punctata* has been implicated as an Ap-variant 1 vector in the absence of *I. scapularis* or *I. ricinus* (Macleod 1962). Ap-infected *Dermacentor albipictus* have been collected from white-tailed deer in Minnesota and are the only tick species currently shown capable of Ap transovarial transmission (Baldridge, Scoles et al. 2009). These species may all play a role in the enzootic

transmission of Ap but due to their host preferences, some are likely transmitting Ap-variant 1 (*H. punctata* and *D. albipictus*) and they are all unlikely to feed on humans so they are less of a public health concern (but see Goddard 2002).

Since Ap is not vertically transmitted in *I. scapularis*, these ticks must acquire the pathogen by feeding on an infected host (Lewis 1979; Munderloh and Kurtti 1995; Ogden, Bown et al. 1998). Ixodid ticks are considered host generalists, meaning they feed on a wide range of mammals, reptiles and birds during their life cycle. Larvae and nymphs typically feed on small mammals or birds (Anderson 1989; Hamer, Tsao et al. 2010), adults typically feed on larger mammals such as deer and all three stages can use humans as incidental hosts (Keirans, Hutcheson et al. 1996).

Reservoirs

Ap ecology differs between Ap-ha and Ap-variant 1 strains. Ap-variant 1 has been found primarily in white-tailed deer *(Odocoileus virginianus)* in the US and has also been shown to be infectious to goats (Massung, Mather et al. 2006). Furthermore, Ap-variant 1 has been shown incapable of infecting mice, even severe combined immunodeficient (SCID) mice (Massung, Priestley et al. 2003), reinforcing the hypothesis that Ap-variant 1 is maintained primarily in ruminant populations (Massung, Mauel et al. 2002).

Early studies reported deer as main reservoirs for Ap-ha, (Massung, Courtney et al. 2005; Reichard, Blouin et al. 2009), however, these studies did not differentiate between Ap strains and thus were likely detecting Ap-variant 1 (Walls, Asanovich et al. 1998; Magnarelli, Ijdo et al. 1999; Courtney, Dryden et al. 2003). Subsequent research

has shown that deer can be exposed to and develop infection from Ap-ha (Tate, Mead et al. 2005; Reichard, Blouin et al. 2009) but deer cannot transmit the infection to feeding ticks (Reichard, Blouin et al. 2009) and are thus not a competent reservoir.

White-footed mice (*Peromyscus leucopus)* are often cited as the main reservoir for Ap-ha, and though mice are competent reservoirs (Telford, Dawson et al. 1996; Stafford, Massung et al. 1999; Levin, Nicholson et al. 2002), recent work has shown that raccoons (*Procyon lotor*) and squirrels (*Sciurus carolinensis*) likely contribute more to Ap transmission. Raccoon and squirrels both host greater numbers of ticks than mice do and they transmit Ap at higher rates than mice (Levin, Nicholson et al. 2002). Stray cats (*Felis domesticus*) and eastern chipmunks (*Tamias striatus*) are also capable of transmission, though due to small sample sizes, more work should be done to evaluate significance of their reservoir competence (Levin, Nicholson et al. 2002). Virginia opossums (*Didelphis virginiana*) and striped skunks (*Mephitis mephitis*) host larval and nymphal ticks but transmit Ap at a very low rate, resulting in infection prevalences lower than that of questing nymphs at the same location. Eastern cottontail rabbits (*Sylvilagus floridanus)* are frequently infected with Ap; one study showed 27% active infection and 66% seroprevalence (Goethert and Telford 2003). Rabbits, however, typically transmit to *I. dentatus* ticks (Goethert and Telford 2003); their reservoir competence for *I. scapularis* is unknown. Reptiles (Nieto, Foley et al. 2009) and pheasants (*Phasianus colchicus;* (Ogden, Bown et al. 1998) have been shown to become infected with Ap-ha but do not appear to be suitable reservoir hosts.

A recent paper found that some species of birds (robins and veeries) may be capable of transmitting Ap to larval ticks but it appears that other birds (grosbeaks and wood thrush) are less capable reservoirs (Daniels, Battaly et al. 2002).

BIRDS IN THE EMERGENCE OF *ANAPLASMA PHAGOCYTOPHILUM*

I. scapularis is increasing its range in the US, particularly in the Midwest (Riehle and Paskewitz 1996; Hamer, Tsao et al. 2010). This expansion is believed to be a result of the long-distance migration of birds carrying feeding ticks and the shorter movements of deer and other mammals (Weisbrod and Johnson 1989; Riehle and Paskewitz 1996; Madhav, Brownstein et al. 2004). Numerous studies have investigated migratory birds as dispersal agents for emerging diseases (Anderson, Johnson et al. 1986; Weisbrod and Johnson 1989; Olsen, Duffy et al. 1995; Olsen, Jaenson et al. 1995; Riehle and Paskewitz 1996; Kurtenbach, Sewell et al. 1998; Brinkerhoff, Folsom-O'Keefe et al. 2011) and birds have been suggested as dispersal agents for Ap.

The minimum necessary stipulations for a bird to be an agent of dispersal for a tick-borne pathogen are that the bird must be a) exposed to the vector, b) exposed to the pathogen, c) able to deposit infectious vectors in suitable habitat and d) able to fly/migrate while infectious. Furthermore, for a bird to play a role in the transmission ecology of a pathogen, it also must a) be able to transmit the pathogen to a naïve vector and b) be infectious for long enough to bridge phenological emergence gaps. No studies have comprehensively addressed these questions but we will address the existing evidence and the likelihood that birds are involved in Ap emergence.

Exposure

I. scapularis is the most common tick found on birds in areas where *I. scapularis* is endemic (Spielman, Clifford et al. 1979; Anderson, Johnson et al. 1986; Battaly, Fish et al. 1987; Anderson 1991; Hamer, Tsao et al. 2010); *Haemaphysalis leporispalustris* and *I. dentatus* are more common where *I. scapularis* is absent (Sonenshine and Stout 1970). *I. scapularis* nymphs and larvae have been found feeding on many different bird taxa, of which, species in the mimidae and thrush families (Magnarelli, Stafford Iii et al. 1992; Ogden, Lindsay et al. 2008), including American robins (*Turdus migratorius)* and gray catbirds (*Dumetella carolinensis),* are frequent hosts (Anderson, Johnson et al. 1986; Battaly, Fish et al. 1987; Anderson 1989; Olsen, Jaenson et al. 1995; Smith Jr, Rand et al. 1996; Hamer, Tsao et al. 2010). Furthermore, Ap-infected nymphs have been removed from many species of birds so they are exposed to both the vector and pathogen (Bjoersdorff, Bergstrom et al. 2001; Ogden, Lindsay et al. 2008; Hildebrandt, Franke et al. 2010).

Transmission

One study found Ap-infected larvae feeding on birds, suggesting that they are not just capable of dispersing infected vectors, but may be capable of transmitting the infection to naïve ticks (Daniels, Battaly et al. 2002). This is circumstantial evidence, however, and other studies have suggested that larval ticks pick up the infection by co-feeding (Alekseev, Dubinina et al. 2001) and that birds cannot transmit systemically (Skotarczak, Rymaszewska et al. 2006). However, even if birds are transmitting at low rates, or only allow transmission by co-feeding, if the population of birds is dense, and

they are heavily parasitized, their contribution to maintaining a pathogen or expanding its range could be substantial (Ginsberg, Buckley et al. 2005; Brown and O'Brien 2011). However, this does not appear to be the case and compared to other *I. scapularis* hosts, birds are not heavily parasitized (Hamer, Tsao et al. 2010).

The other option for transmitting the pathogen to naïve larvae is via co-feeding, which has been shown to occur with other pathogens (Randolph, Gern et al. 1996). However, since birds are not heavily parasitized, they would be highly unlikely to transmit significantly via this route. The ability of birds to transmit Ap systemically and via co-feeding needs to be addressed in captive studies.

Migrating while exposed

A unique aspect of tick-borne diseases is that not just the infection but also the infected vector can be carried by a bird during migration. As long as the pathogen does not affect the bird's mobility, and is transmitted around the time when birds are migrating, birds would be capable of acting as a dispersal agent. This is particularly true for long-distance migrants like migrating seabirds, which have been implicated in the transhemispheric dispersal of *B. garinii*-infected ticks (Olsen, Duffy et al. 1995; Benskin, Wilson et al. 2009). With any emerging zoonotic pathogen, there is the concern of how it will impact the health of its host, and in the case of birds, avian conservation attempts. This could profoundly affect a bird's potential as a dispersal agent; if a pathogen makes its host very ill, the host can become less mobile or immobile and limit the spread of the pathogen (except in cases with highly mobile vectors).

Ap infection typically causes fever in infected equine (Gribble 1969), bovine (Tuomi 1967), ovine (Woldehiwet 1987), canine (Scorpio, Dumler et al. 2011), and human hosts (Dumler, Choi et al. 2005). Fever can be energetically expensive; a rise in temperature of 1°F can increase energy expenditure 7 per cent of basal metabolic value (DuBois 1921). If infection or trauma are the cause of the fever, the energy expenditure cost is particularly high (Roe and Kinney 1965) and can result in behavioral changes and weight loss (Baracos, Whitmore et al. 1987; Bonneaud, Mazuc et al. 2003; Adelman and Martin 2009). Thus, if Ap infection results in fever, it may affect a bird's ability to migrate during infection and thus the bird's ability to disperse a pathogen; this has been shown to occur with West Nile virus (Owen, Moore et al. 2006) and needs to be investigated for Ap.

Depositing infected vectors into viable habitats

For this condition to be satisfied, birds would have to share suitable habitat with *Ixodes* ticks. Suitable habitat would be considered one that has sufficient climate, hosts, reservoirs etc. to support a tick population and sustained Ap transmission. Moreover, for such a focal tick population to become established, birds would have to deposit ticks with sufficient frequency to start an effective breeding population.

Many bird species share habitat with *Ixodes* ticks, as demonstrated by the frequent infestation of birds with such ticks (Spielman, Clifford et al. 1979; Anderson, Johnson et al. 1986; Battaly, Fish et al. 1987; Anderson 1991; Hamer, Tsao et al. 2010). Building on this, migrating birds have been implicated in the expansion of *I. scapularis* range (Hamer, Tsao et al. 2010), and the distribution of Ap (Bjoersdorff, Bergstrom et al. 2001; Ogden,

Lindsay et al. 2008) and other tick-borne pathogens (ex: Bb; (Scott, Fernando et al. 2001; Brinkerhoff, Folsom-O'Keefe et al. 2011), though it is difficult to study this question empirically. Furthermore, migratory birds are frequently found in habitats surrounding human dwellings, areas that are often also suitable for *Ixodes sp.*, making birds ideal for bringing the infection or infected vectors into proximity of humans (Ginsberg, Buckley et al. 2005).

However, the ability of birds as dispersal agents is contingent upon their ability to transmit the infection to naïve larvae. Birds have been found migrating with infected nymphs on them and therefore, they likely would drop an engorged, infected nymph off in a new location. This would be unlikely to start a new infection foci, however, since the nymph would molt into an infectious adult, who would then likely feed on a deer (Lane, Peisman et al. 1991); since deer are not reservoirs (Reichard, Blouin et al. 2009) of public health importance (Tate, Mead et al. 2005) even an exposed deer would be unlikely to pass along the infection.

Tick phenology and transmission dynamics

Ap is not vertically transmitted in *Ixodes* ticks (Lewis 1979; Munderloh and Kurtti 1995); therefore even if birds are capable of maintaining an infection like Ap, to play a role in transmission they need to maintain the pathogen for long enough to pass it from infected nymphs to the naïve larvae. This is a critical factor, especially in the Northeast where nymphal and larval emergence peaks are separated by approximately eight weeks (Gatewood, Liebman et al. 2009). However, in the Midwest, nymphal and larval activity peaks overlap significantly (Gatewood, Liebman et al. 2009) such that

during the peak of larval activity, there are still significant numbers of nymphs feeding on and seeking hosts. This overlap would allow for infection of larvae despite a transient infection (Davis and Bent 2011) and could potentially have far-reaching effects on strain type and pathogenicity (Gatewood, Liebman et al. 2009).

INTERACTIONS WITH *BORRELIA BURGDORFERI*

Due to the shared ecology of Bb and Ap, there is ample opportunity for coinfections and interactions. These interactions between Ap and Bb could occur both in the host and the tick vector. In the vector, there does not appear to be any interaction with respect to acquisition and transmission: previous infection with either pathogen does not hinder the acquisition of the other pathogen (Levin and Fish 2000) and coinfection with these pathogens does not affect transmission success of either or both pathogens; dually infected ticks are capable of transmitting one or both pathogens at the same rate that singly-infected ticks transmit the infection (Levin and Fish 2000).

In the host, however, the way coinfection impacts infection and transmission may depend on how coinfections were acquired and their order of infection. In simultaneous, needle-inoculated mice (*Mus musculus* C3H), coinfection increases bacterial burden and transmission of both pathogens and increases the severity of arthritis caused by borrelial infection (Thomas, Anguita et al. 2001). This is not surprising, given that Ap was originally identified because coinfection with Ap increased the tick-borne fever fatality rate (Batungbacal, Scott et al. 1982; Gilmour, Brodie et al. 1982). However, these results contrast sharply with the simultaneous coinfection results of Levin and Fish (2000), who found there to be no interaction between the two agents as far as transmission.

In sequential infections, the outcome can also be ambiguous. When mice were infected with Bb first, then Ap a week later, they transmitted Ap to naïve ticks at a lower rate than if they were singly infected with Ap (Levin and Fish 2001). The reverse, however was not true; sequential infection with Ap then Bb did not affect mouse to tick Bb transmission (Levin and Fish 2001). This is slightly counter-intuitive; since Ap infects macrophages and can lower white blood cell counts, one might expect prior Ap infection to facilitate the transmission of a secondary infection. Indeed we see this in clinical situations where HGA patients can fall victim to a second infection (Bakken, Krueth et al. 1996; Dumler, Choi et al. 2005). Another study done on free-living reptiles supported the finding that prior Ap infection can limit Bb (Vaclav, Ficova et al. 2011). However, this also found an overall positive effect of Bb on Ap which led to higher than expected probability of coinfection in tick vectors (Vaclav, Ficova et al. 2011). The differences between these two studies may prove to be due to host-specificity.

CONCLUSIONS

Birds satisfy all the conditions to act as dispersal agents of Ap however, to determine the role birds play in Ap transmission and emergence, a number of important issues need to be tested empirically. Most fundamental of these issues is the unknown reservoir competence of birds. Studies assessing this possibility should focus on abundant, migratory species that are frequently infested with *I. scapularis* ticks since such species would likely be most important for Ap transmission and dispersal ecology. Furthermore, determining the health impact of Ap infection on such species is also critical as severe illness could preclude them from migrating. Once these two issues have been resolved, research can address more specific questions such as: the variation of

reservoir competence and exposure among species, the effect of coinfection and immunosuppressive life-history events on transmission, and the frequency with which infected ticks are deposited in suitable habitats.

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CHAPTER 2

ANAPLASMA PHAGOCYTOPHILUM INFECTION IN TWO SPECIES OF PASSERINE BIRD, AMERICAN ROBINS AND GRAY CATBIRDS: AN ASSESSMENT OF RESERVOIR COMEPTENCE AND DISEASE

INTRODUCTION

Anaplasma phagocytophilum (formerly *Ehrlichia phagocytophilum* and *Equine phagocytophilum*), is a tick-borne bacterium and the causative agent of Human Granulocytic Anaplasmosis (HGA), a disease which affects over 1000 US citizens each year (Dumler, Barbet et al. 2001; CDCP January 2011). *A. phagocytophilum* (Ap) was originally identified in Scotland in 1940 (Gordon, Brownlee et al. 1940) as a pathogen of ruminants and was considered as such until 1994 when a Wisconsin man fell ill with HGA, marking the first case of Ap in the US and the first incident of Ap infecting a human (Chen, Dumler et al. 1994). Since its discovery in the US, Ap has been expanding its range and HGA prevalence has been increasing, making it an important emerging infectious disease and the second only to *Borrelia burgdorferi* (Bb), the bacterium that causes Lyme disease, as the most common tick-borne pathogen in the Northern Midwest and Atlantic Seaboard regions (CDCP January 2011).

There are two main strains of Ap in the US: Ap-variant 1 and Ap-human agent (Ap-ha) which vary in their transmission ecology (Massung, Mauel et al. 2002). Both strains use *Ixodes scapularis* (*I. scaularis*), as the main vector but they use different reservoir hosts to maintain transmission. Ap-ha, the strain that causes HGA, uses small to medium-sized mammals as reservoirs and deer are an incompetent or sub-optimal host (Levin, Nicholson et al. 2002; Reichard, Blouin et al. 2009). Conversely, Ap-variant 1

does not infect mice nor humans and appears to use deer and other ruminants as its main reservoirs (Massung, Priestley et al. 2003; Reichard, Blouin et al. 2009).

The importance of birds in Ap ecology is still unknown; larval and nymphal stages of *I. scapularis* ticks feed on birds (Anderson 1991; Hamer, Tsao et al. 2010), and Ap-infected nymphs have been removed from birds (Bjoersdorff, Bergstrom et al. 2001; Skotarczak, Rymaszewska et al. 2006; Hildebrandt, Franke et al. 2010). A recent study found *A. phagocytophilum*-infected larvae on two species of bird: American robins (*Turdus migratorius)* and veerys (*Catharus fuscescens*). Since Ap is not vertically transmitted in *I. scapularis* ticks (Lewis 1979; Munderloh and Kurtti 1995; Ogden, Bown et al. 1998), this finding suggests that these species may be infectious (competent) reservoirs. Captive studies are needed to assess this possibility and what role, if any, birds play in the natural transmission dynamics of Ap.

Another understudied area in many tick-borne zoonotic pathogens is how they affect the health of their avian host. Bb has not been shown to cause disease in birds (Bishop, Khan et al. 1994), but other tick-borne pathogens, such as *B. hermsii* (Thomas, Bunikis et al. 2002) are known to do so. Yet, few studies have looked for signs of subclinical infection which, though more difficult to detect in wild animals, could still impact their behavior and fitness (Owen, Moore et al. 2006). No studies have assessed whether birds develop an analogue to HGA or tick-borne fever.

In this study we experimentally tested the Ap reservoir competence of two common, migratory bird species: American robins and gray catbirds (*Dumetella carolinensis).* Both species are frequently parasitized by larval and nymphal *I. scapularis* ticks (Battaly, Fish et al. 1987; Ogden, Lindsay et al. 2008; Hamer, Tsao et al. 2010) and

are therefore likely exposed to Ap. We predicted that these two species would vary in their reservoir competence for Ap, due to previous studies on other pathogens. For instance, American robins are more permissive of Bb (Mather, Telford et al. 1989; Ginsberg, Buckley et al. 2005), *Plasmodium relictum* (Beaudoin, Applegate et al. 1971) and West Nile virus (WNV; (Komar, Langevin et al. 2003; Kilpatrick, LaDeau et al. 2007) while gray catbirds (*Dumetella carolinensis*) are more resistant to those same pathogens as indicated by levels of viremia/bacteremia or transmission. We use the term resistance to refer to any host strategy (or host) that effectively limits infection (Roy and Kirchner 2000; Raberg, Graham et al. 2009). Furthermore, we predicted that the varying responses to the pathogen would result in variation in the quantitative signs of disease (fever, weight loss) exhibited by the infected birds. These signs of disease were separated from the effects of tick parasitism in the experimental design via three treatments: birds were exposed to infected ticks, uninfected ticks or no ticks. Assessing avian reservoir competence will improve our understanding of Ap transmission ecology, which has implications for public, wild and domestic animal health. Understanding the health impact that this pathogen has on birds will also be important for avian conservation efforts.

MATERIALS AND METHODS

Focal species

Wild catbirds (n=13) and robins (n=30) were captured using mist nets (12 x 2.6 m with 30 mm mesh) at several locations in central Michigan (USFWS Scientific collection permit #MB194270-1, Michigan State Scientific Collector's Permit #SC-1386, Michigan

Department of Natural Resources special-use permit #09-RL-03-1, Institutional Animal Care and Use Committee protocol 01-10-007-00). We targeted hatch year birds in areas where *I. scapularis* ticks have not been detected (Hamer, Tsao et al. 2010) to minimize the likelihood of birds being previously exposed to Ap. Birds were then immediately transported to the University Research Containment Facility at Michigan State University, housed in individual cages (18"L x 18"D x 24"H, 5/8" wire spacing), with a 12:12 light:dark cycle and given water *ad libitum.* A blood sample (200ul from catbirds and 400ul from robins) was taken upon entry and once a week during captivity from the brachial vein.

Birds were fed a mixture of blueberries, cottage cheese, crickets, wheat and barley with live mealworms and moistened monkey biscuits (see Supplementary Materials for recipe). Since body condition is known to affect immune function (Owen and Moore 2008) and these species are known to over-eat in captivity (Owen pers. comm.), we restricted their diet to maintain a natural body condition and standardize daily food intake. Four Balb/C mice (*Mus musculus;* Harlan Laboratories, Haslett, MI) were simultaneously exposed as positive controls. Mice were provided with food and water *ad libitum* and housed in cages with wood-chip bedding. Bird cages were randomly arranged on racks with three rows of shelves, three cages to a shelf so that birds were not visually or aurally isolated from one another.

Experimental design and exposure

Laboratory-raised Ap-infected (Dawson strain; approximately 40% prevalence of infection) and uninfected *I. scapularis* nymphs were purchased from the US Center for

Disease Control and Prevention (Levin and Ross 2004). An incomplete randomized design was used where robins were randomly assigned to control (RobCon; n=10), tick (RobTic; n = 10), or Ap-exposed (RobExp; n = 10) groups. Catbirds were randomly assigned into two groups; tick (CatTic; n=3) and Ap-exposed (CatExp; n=10). Treatment differences only occurred on exposure day; from 1 day post-exposure (1 dpe), all birds were treated similarly. On 0 dpe, birds in Ap-exposed and tick groups had 10 infected or 10 uninfected (respectively) nymphs brushed onto their head and neck. Control birds had no ticks attached to them. All birds were then placed into individual restraint chambers for approximately 4 hours in a dark, quiet room to minimize grooming and allow ticks time to attach. Chambers were made of round polyvinyl chloride pipe (8" X 3" for robins, 8" X 2" for catbirds) with valence material covering each end. At the end of the attachment period, chambers were checked for ticks and birds were returned to cages. If any nymphs or over five larvae were found in the restraint chamber, they were brushed back onto the bird and the bird was returned to the restraint chamber for an additional hour. If less than five larvae were found, they were killed in a bleach solution. Beneath each cage was a water-filled pan, to catch engorged ticks. Pans and mouse cages were lined with petroleum jelly to prevent ticks from crawling out. Carpet protection sticky tape was placed sticky-side up under all cages and wrapped around the outside of each shelving unit to trap any ticks that did not drop into water pans. During the 0 dpe exposure period, it appeared that ticks were being flung outside of the host's cage, since ticks were found stuck to the carpet protection tape and three ticks were found in water pans of control birds that had not been infested with ticks. Clear plexiglass tiles (24" X 24") were placed in between cages to prevent tick flinging without visually isolating the

birds. Mice were anesthetized with ketamine (80 mg/kg) and xylazine (16 mg/kg) during infestation with ticks. Ten infected nymphs were brushed on the head and neck of the mice; mice were housed in restraint chambers (50ml conical tube with valance covering the open end) until the anesthesia wore off (approximately one hour) and then returned to cages. During infestation periods, mice were housed in cages with wire mesh over water for collection of engorged ticks.

Tick collection

Water pans, petroleum jelly and carpet tape were checked beginning at 1400 hrs daily for 8 days following tick attachment. Engorged ticks were collected, sterilized in 10% bleach, then rinsed in water and measured for maximum length and width (nymphs only) and mass (larvae in groups of 5). Ticks were then placed in 12 x 75mm polystyrene culture tubes with mesh tops and kept at room temperature and 95% relative humidity in humidity chambers containing a saturated solution of magnesium sulfate. Humidity chambers were kept in the same room as the birds on a 12:12 light cycle. Tubes were checked frequently for molted ticks, which were removed and put into 70% ethanol. Unmolted ticks that appeared dead after a minimum of 42 days were removed and put in ethanol to minimize deterioration and fungal growth.

Xenodiagnoses, DNA extraction and quantitative polymerase chain reaction

Xenodiagnosis is the process of diagnosis by which the host is exposed to the vector and then vector is then tested for the agent (Donahue, Piesman et al. 1987; Randolph, Gern et al. 1996; Goethert and Telford 2003). In this case, we attached larval

ticks to determine if the hosts were infectious. Xenodiagnoses were performed on 7, 14, 42, and 77 dpe. Pathogen-free larvae (source – Durland Fish, Yale University; n=30-60) were attached to each bird, collected and stored using the same protocol as described for nymphs.

Since Ap replication is activated by feeding (Hodzic, Fish et al. 1998), all ticks with detectable Ap DNA are considered capable of transmitting Ap (Levin and Fish 2000; Ross and Levin 2004); thus, infected molted ticks would be considered capable of transmitting. Furthermore, molted and unmolted ticks were treated equally in analysis since infection with the Dawson strain of Ap has been shown to have no effect on tick molting success (Ross and Levin 2004). DNA was extracted using a DNEasy Blood and Tissue Extraction Kit (Qiagen Inc., Valencia, CA). Manufacturer instructions were followed unless otherwise noted; ticks were frozen in liquid nitrogen and crushed using grinding pestles and allowed to sit overnight with proteinase K solution. DNA was purified and eluted the next day in 50ul AE buffer. Ap infection was determined by quantitative polymerase chain reaction (qPCR) targeting the 16S rRNA gene (Massung, Priestley et al. 2004). In initial qPCR runs, some known negative samples came up positive for Ap. All false positives gave a weak signal with a cycle threshold value between 35-40 so all positive samples with a cycle threshold value between 30-40 were re-tested. After buying new primers and probe we had no further false positive or contamination issues. All ticks to a minimum of 12 were tested from each bird from a particular xenodiagnosis. At least 12 ticks were tested from each control and tick treatment bird to confirm that they were not transmitting Ap. All samples were run in duplicate and discrepant results were retested until a majority was reached.

Bacteremia

Blood from birds was collected on days -14, -9, -7, -5, -3, 0, 3, 5, 7, 9, 14 dpe via the brachial vein and from mice via the tail vein on 7, 14 and 21 dpe. These time points were chosen as the best time to detect the kinetics of the infection (Massung, Priestley et al. 2004). Blood was collected directly into a homemade RNA preservative (1:5 ratio; see Supplementary Materials for recipe) to preserve RNA for a separate study and stored at -80°C until use. Blood used for bacteremia assays was then centrifuged at 8000 rpm for 1 minute and supernatant was discarded. DNA was extracted from the remaining cell pellet following the cell culture protocol for the DNEasy blood and tissue extraction kit. Infection was determined via qPCR as described for ticks and double stranded DNA was quantified according to manufacturer's directions using the Picogreen quantitation kit so bacteremia results could be standardized (Ap copies/ug DNA; (Massung, Priestley et al. 2004).

Serology

Blood for serological testing was collected via the brachial vein from each bird upon entering captivity, on 14 dpe for all birds euthanized that day, and day 28 dpe for all other birds. Mouse blood from 84 dpe was used to confirm antibody presence since these were the only serum samples available that were not stored in RNA preservative, since the preservative was found to interfere with the assay (unpublished data).

Blood was allowed to clot at room temperature, spun down and the serum and cell pellet were stored separately at -80°C. All serological analysis was conducted through the

Diagnostic Center for Population and Animal Health (East Lansing, MI). Detection and titration of Ap-specific antibody was done using indirect fluorescent antibody (IFA) staining. A commercially available fluorescein isothiocyanate conjugated goat anti-wild bird IgG or fluorescein isothiocyanate conjugated goat anti-mouse IgG (both conjugates were reactive against heavy and light chains of IgG [Bethyl Laboratories, Montgomery, TX]) were used to detect antibody bound to commercially available substrate slides coated with the Martin isolate of Ap (VMRD, Pullman, WA). High serological cross-reactivity has been shown among Ap strains (Dreher, De La Fuente et al. 2005), making this assay broadly useful for detecting Ap antibodies.

The bird and mouse sera were diluted 1:5 in PBS (pH 7.4). From those starting dilutions of sera, serial two fold dilutions were made in PBS through 10,240. The diluted sera were applied to the substrate slides and incubated for 30 minutes at 37° C in a humidified chamber. Briefly, the anti-wild bird and anti-mouse IgG conjugates were diluted 1:10 in a 0.1% w/v solution of Evans blue in phosphate buffered saline (PBS, pH 7.4) before use. After incubation, the substrate slides were washed thoroughly with PBS and the diluted anti-wild bird or anti-mouse conjugates were applied to the slides. The slides were again incubated for 30 minutes at 37° C and then washed thoroughly. A coverslip was added to each slide. The slides were examined, using a fluorescence microscope and a 40X objective, to identify the last dilution of sera which showed fluorescent staining of the bacterium. The positive control used for the test was a known positive Ap-specific IgG antibody in equine serum with an appropriate conjugated antibody against equine IgG. The negative control used for the slides was serum from a turkey raised in confinement and each of the conjugates applied to slides without

previous application of bird or mouse test sera. The optimal dilution of anti-wild bird conjugate was determined using a known positive wild bird serum against West Nile virus on substrate slides made in-house from Vero cells infected with West Nile virus. The optimal dilution of anti-mouse conjugate was determined using a known positive mouse serum against Bb and a substrate slide for that bacterium.

Host health

On each sampling visit (see Bacteremia, plus days -11, 11, 17, 19, 21 dpe) birds were weighed (nearest 0.1 g) and body temperature (to nearest 0.01ºC) was recorded. Body temperature was measured via the cloaca using a Roetemp TM99-A thermometer and a 30g Type K thermocouple (Roetemp, San Diego, CA, USA). Temperature readings were recorded after 10 seconds (temp A) and again once the temperature reading had stabilized for 5 consecutive seconds (temp B). Temp B was used in the analysis since the method was more biologically relevant and the results were consistently within the expected range for this species. The tip of the thermometer was wiped with 70% isopropyl alcohol and allowed to dry between individuals. Temperature was taken at the same time of day due to diel fluctuations and in the same order to control for ordinal effects, which, when tested for, were not statistically significant (data not shown).

Euthanization and necropsy

Birds were euthanized via CO_2 asphyxiation and necropsied on 14, 21 or 100 dpe depending on group designation; half of each treatment and all birds in the CatTic treatment were euthanized at 14 dpe, the remaining RobCon birds were euthanized at 21

dpe and all remaining birds were euthanized after the 77 dpe xenodiagnosis. Mice were euthanized via CO_2 asphyxiation and necropsied at 84 dpe. Blood was collected via cardiac puncture for mice and via jugular vein for birds. Tissue samples from the spleen, heart, liver, kidney, and lung were sterilely collected and stored at -80ºC for this and other studies. Liver and spleen samples were split into two, with half stored at -80ºC and half preserved in formalin for 48 hours and then transferred to ethanol for storage at room temperature.

Data analysis

For tick infection prevalence analysis, although data was collected at the level of the individual tick, treatments and repeated measures were at the level of host thus the percentage of infected ticks per bird was used as the response variable. This data violated the assumptions of independence and equal variance across time so we used a mixed model with independent variance (repeated measures structure) to account for these violations (Littell, Henry et al. 1998). Since the experiment was not balanced, denominator degrees of freedom were calculated using the Welch–Satterthwaite procedure. An autoregressive covariance structure was selected based on Akaike information criteria score. In the mixed model, species and xenodiagnostic event were predictor variables and bird identity was included as a random effect. Residuals for the full mixed model were normally distributed (Shapiro-Wilk, $p>0.05$). The estimate of least squares means for almost all birds during xenodiagnostic events was zero, rendering an analysis of variance ineffective for valid statistical comparisons. When transmission was

not zero, we reported the mean transmission ± one standard error. We used an analysis of variance (ANOVA) to compare differences among groups for Ap exposure at 0 dpe.

For comparisons of temperature and mass change, we used the same statistical model, using species, treatment group and study period as predictor variables. Study periods were separated into pre-exposure (-14, -11, -9, -7, -5, -3, 0 dpe), acute (3, 5 dpe) and post-exposure (7, 9, 11, 14, 16, 19, 21 dpe). Since birds were not bacteremic nor transmitting at 7 dpe, any immune response to Ap exposure would have occurred during the 3 and 5 dpe observations, so this was designated as the acute infection period. There was a treatment by period interaction for both mass (F_{246}=5.32 p<0.001) and temperature (F_{272}=3.58, p=<0.001), thus we isolated species and compared groups within treatment or period to test all *a priori* hypotheses. All other post-hoc pairwise comparisons were assessed using a Tukey-Kramer correction to account for the increased type I error rate associated with multiple comparisons. Robins weigh approximately twice as much as catbirds so mass change was reported as percent change from initial mass (taken on -14 dpe). All analyses were performed using SAS version 9.2 (SAS Institute Inc, Cary, NC) using an alpha value of 0.05.

RESULTS

Anaplasma phagocytophilum *pathogen exposure*

There was no significant difference in the prevalence of Ap in the 0 dpe post-feeding nymphs (engorged, unmolted nymphs and nymphs that successfully molted into adults) from robins, catbirds and mice (Figure 1; F_{21}=0.48; p=0.628). All but one of the

birds in the Ap-exposed groups had one or more of the feeding (0 dpe) nymphs test positive for Ap, confirming exposure to the pathogen. Three birds from the tick group; two robins and one catbird had an infected nymph collected from them (Supplement 1). All other 0 and 7 dpe ticks from these birds were tested and no other ticks came up positive.

We were unable to confirm exposure for one robin (R8) in the Ap-exposed group, as we only were able to retrieve and test two fed nymphs on 0 dpe from that individual. Considering there was a 40% infection rate among the nymphs before attachment, and each bird was exposed to 10 ticks, we assume that this bird was exposed and thus have left the ticks in the Ap-exposed treatment group for analysis.

Infected nymphs were collected from the cage of three birds in the uninfected tick group. We tested the rest of the 0 dpe ticks from those birds and no other nymphs were found to be infected. The 7 dpe xenodiagnostic ticks from each bird were tested and none were positive. This suggests that the ticks may have fed on a nearby bird and been flung or crawled to another cage; thus, we assumed that these birds were unexposed and left these three birds in their original groups.

Xenodiagnosis

One robin infected 2 of 13 larval ticks, resulting in 15% transmission for that individual and 1.5%±1% transmission for the species. No other birds were found to transmit at any time (Figure 1). All mice transmitted through day 42 at very high rates (93.8%±4%, 83.1%±25%, 64%±43%, on 7, 14 and 42 dpe, respectively; Fig. 1). No animals transmitted Ap during the 77 dpe xenodiagnosis.

Bacteremia

None of the birds were found to be bacteremic at any time point (3, 5, 7, 9, 14 dpe), but all mice were bacteremic on 7 dpe with an average of 93 copies of Ap 16S rRNA gene (range 6-295) per ug of DNA. None of the mice were bacteremic on 14 dpe but on 21 dpe one mouse was bacteremic with 17 copies per ug DNA.

Serology

None of the birds were found to have Ap-specific antibodies upon entry nor at 14 or 28 dpe. All four mice had antibodies detectable at 84 dpe.

Host Health

Our *a priori* prediction that exposed birds would lose weight was not supported; neither RobExp (t_{269}=-1.16, p=0.248) nor CatExp (t_{97}=1.67, p=0.099) lost weight from the pre-exposure period to the acute period (Figures 2, 3). Post-hoc analysis showed that the RobTic and RobExp groups gained weight from the acute to the post-exposure period though neither was significantly different from the RobCon group during this period (t_{50}=-1.67, p=0.762; t_{51}=-0.39, p=1.000, respectively) and RobExp was not significantly different from RobTic (t_{49}=-1.29, p=0.933).

CatExp experienced a significant increase in temperature from the pre-exposure to the acute period (t_{96}=3.51, p<0.001), which supported our *a priori* prediction of a fever response (Figures 4, 5). However, although CatTic did not experience a significant

increase in temperature (t_{96}=-0.15, p=1.00), CatExp was not significantly different from CatTic during this period (Figure 3; t_{75}=1.60, p=0.598). RobExp did not increase temperature (t_{225}=-1.42, p=0.157); however, it was significantly lower than the RobCon (t_{236}=3.63, p<0.001) though not lower than the RobTic (t_{236}=-1.57, p<0.118) during the acute period.

Figure 1. Average prevalence of infection in exposure nymphs (0 dpe) and xenodiagnostic larvae (7, 14, 42, 77 dpe) post-feeding (molted and unmolted ticks combined) on individuals from the Ap-exposed group in each species.

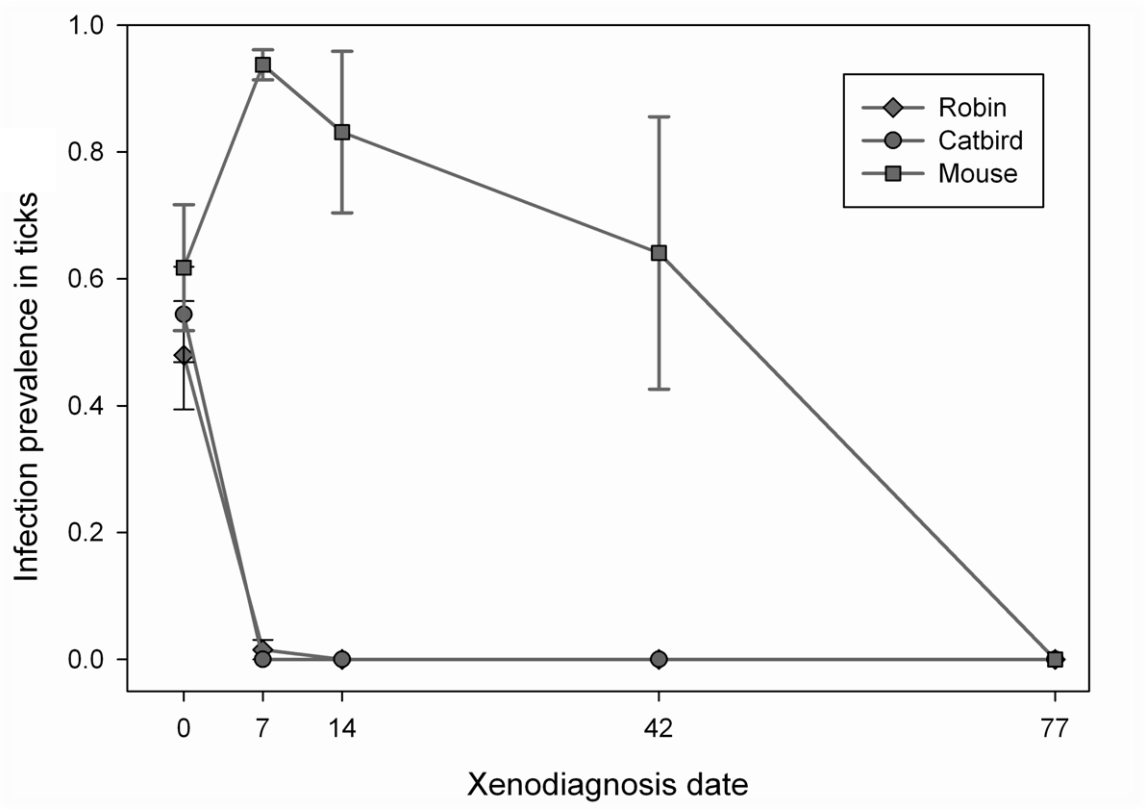


Figure 2. Average relative mass change (± 1 standard error) for American robins per time period.

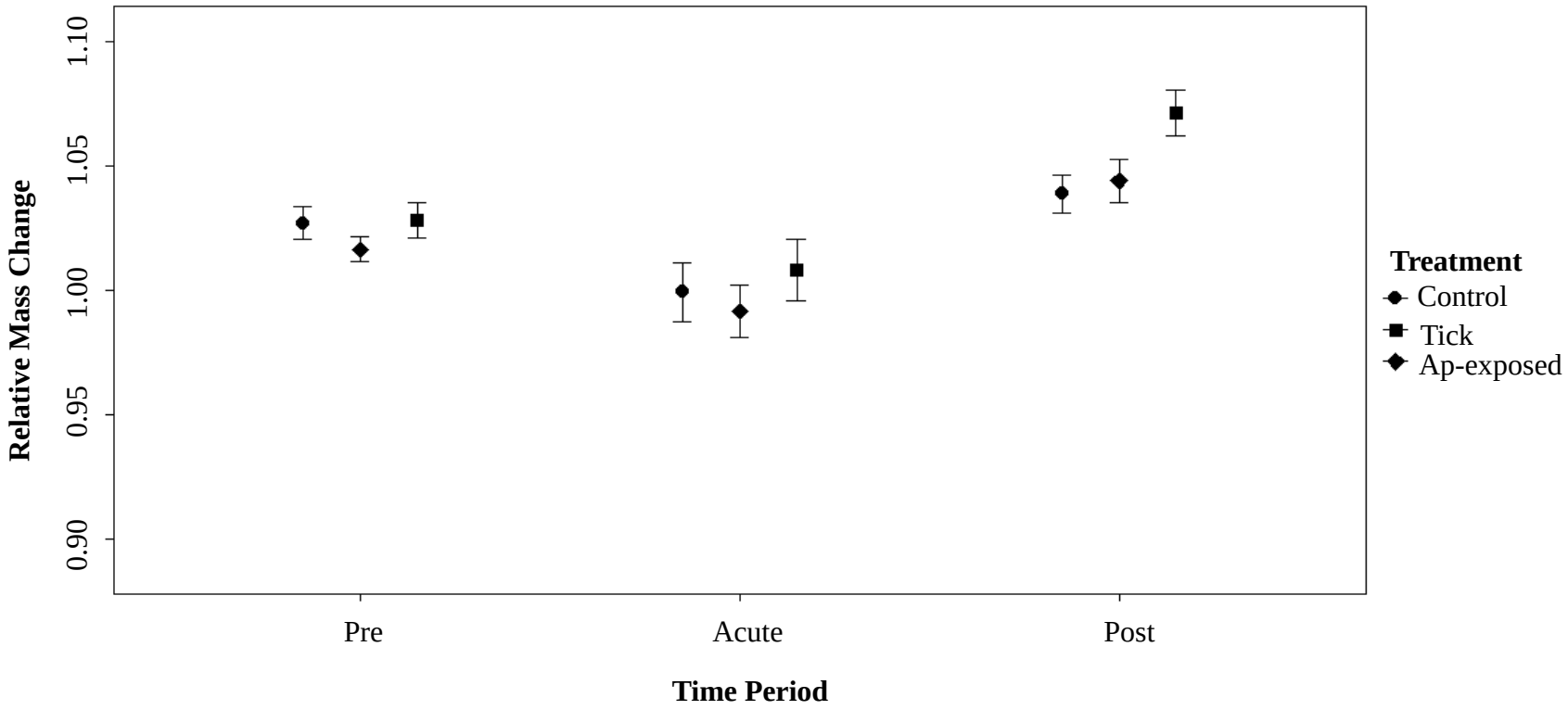


Figure 3. Average relative mass change (± 1 standard error) for gray catbirds per time period.

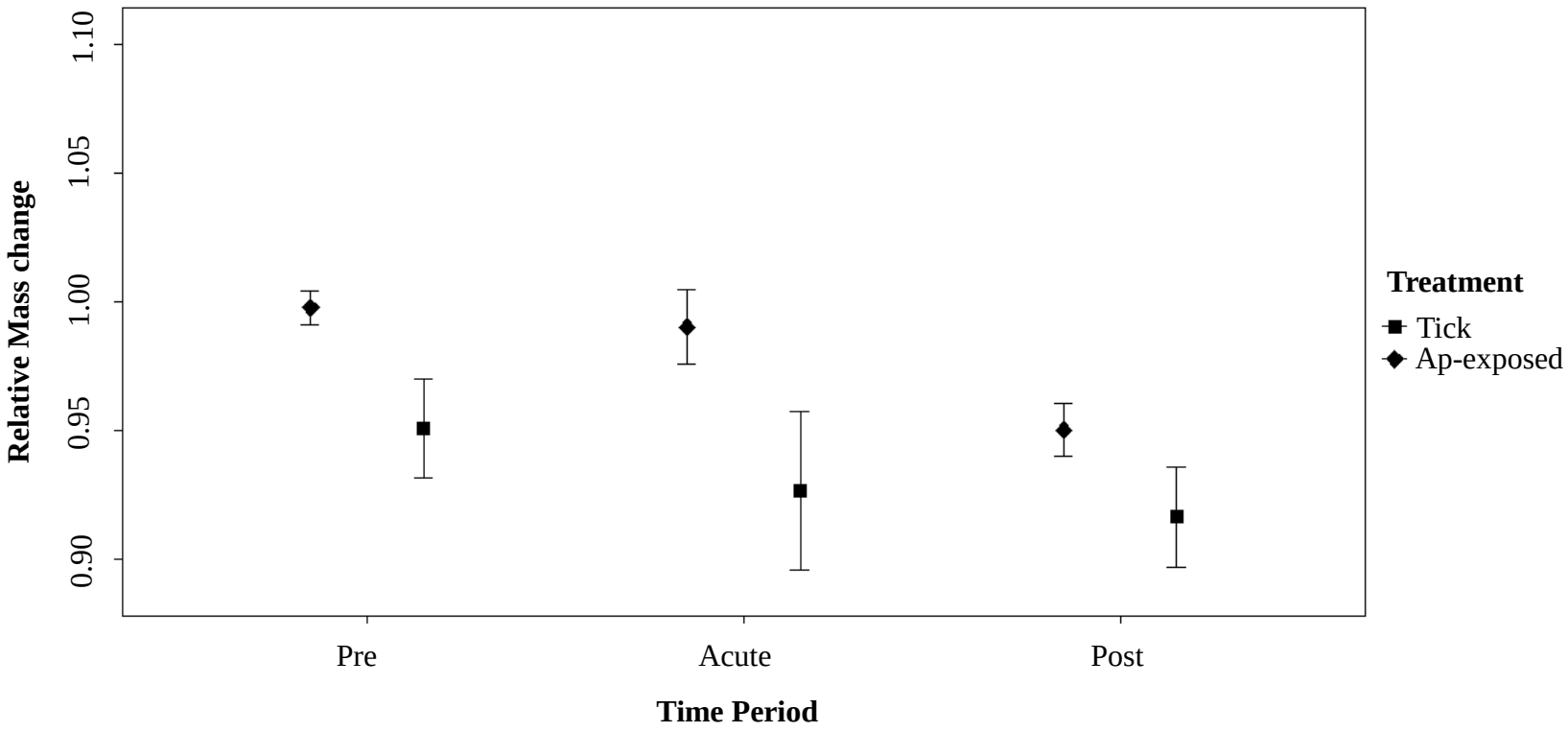


Figure 4. Average temperature (± 1 standard error) for American robins per time period.

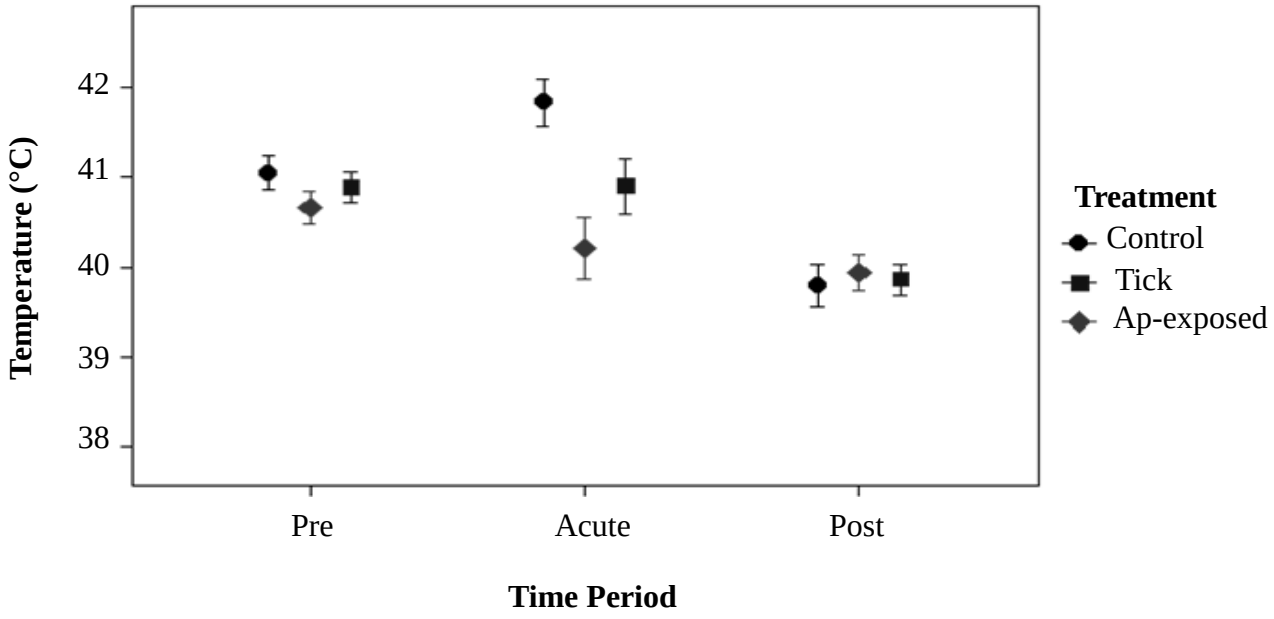
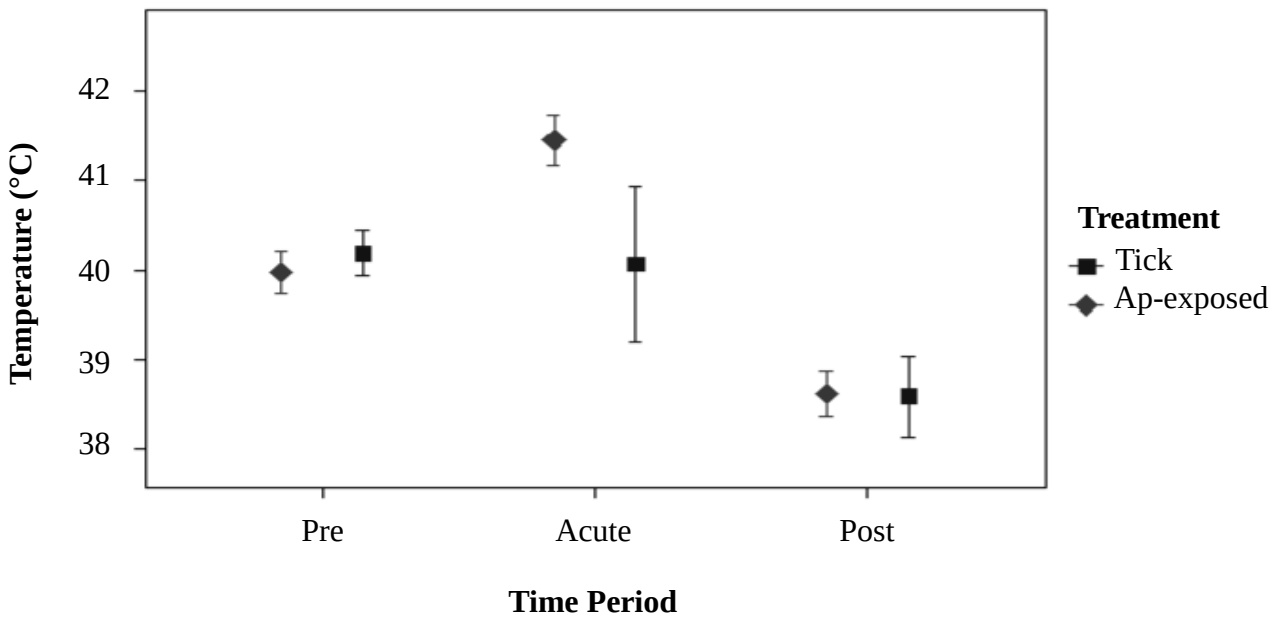


Figure 5. Average temperature (± 1 standard error) for gray catbirds per time period.



DISCUSSION

Our study showed that two common bird species, American robins and gray catbirds, appear to be resistant to infection with Ap. After having 10 infectious *I. scapularis* nymphs (40% infection prevalence) feed on them, none of the birds developed bacteremia on the days tested, and only 1 of 20 exposed birds transmitted Ap to xenodiagnostic larvae. The one robin that transmitted Ap to *I. scapularis* larvae did so at 7 dpe and transmitted to 15% of the feeding ticks. Ap-specific antibodies were not detected in any of the birds. In contrast, all mice were bacteremic on 7 dpe, transmitted at high rates through 42 dpe, and had detectable antibodies at 84 dpe, confirming that our host exposure and Ap assay methods were functional. Therefore, we conclude that catbirds and robins appear to be resistant to infection with Ap.

One mouse was bacteremic on 7 and 21 dpe but not 14 dpe, suggesting recrudescence, which is a characteristic of Ap (Levin and Ross 2004). The four mice and one robin that transmitted the bacteria were able to do so despite undetectable bacteremia (for the bird on 7 dpe and mice on 14 dpe), which supports the conclusion of previous studies that xenodiagnosis is more sensitive than qPCR of blood samples for detecting Ap infection (Levin and Fish 2001; Levin, Nicholson et al. 2002; Goethert and Telford 2003).

Despite the apparent resistance of these birds, we did observe a significant increase (1.3C) in temperature (i.e., a fever response) in the exposed catbirds during the acute exposure period. Fever has been shown to occur in birds in response to an injection with lipopolysaccharide, so immune activation, even in absence of an active infection (Adelman, Cordoba-Cordoba et al. 2010; Moller 2010), which may be what occurred in

the current study. There was no evidence, however, for a fever response in the Ap-exposed robins and although their temperature was significantly different from the control robins during the acute period of exposure, this difference was due to a slight temperature increase for control robins and decrease for exposed robins. I observed substantial variation in temperature throughout this experiment (Supplement 2) which I feel makes the conclusions from the temperature results less robust. Further studies could assess the effect of immune system activation on the catbird fever response.

Activating the immune system and maintaining an elevated body temperature can be energetically demanding (Roe and Kinney 1965; Kluger, Kozak et al. 1998) thus, we would expect to see a corresponding weight loss in exposed birds, particularly those that developed a fever. However, we did not observe weight loss in the exposed birds of either species. This is not surprising for robins since none of the birds developed a detectable bacteremia nor a fever. It is surprising, however, for the exposed catbirds that did exhibit an elevated body temperature, which may suggest that the catbirds were able to compensate for their increased energy demand, or that the observed temperatures were within the normal temperature fluctuations of these birds and did not signify disease. The fact that two of the robin groups increased mass post-exposure suggests that the birds were getting enough calories to accommodate an increased energetic demand. Wild birds however, might not be able to compensate for the increased demand and could experience a fitness impact. Our data does show elevated temperatures in some birds during the pre-exposure period which would support the latter hypothesis though these two hypotheses are not mutually exclusive.

Even in the absence of fever, birds have a higher average body temperature (41ºC on average; (Moller 2010), 40.3 ºC in the current study) than the 37ºC , which is reported as the preferred temperature for Ap growth *in vitro* (Borjesson 2008). Mammals often develop a fever in response to Ap infection that helps kill the bacteria and the natural temperature of birds is similar to the mammalian fever condition which may be inhospitable to the Ap bacterium. A fever response in the birds would exacerbate this effect and further reduce the ability of Ap to infect an avian host. However, other pathogens with a similar growth temperature preference (ex: Bb, WNV) can effectively infect birds so the effect of avian body temperature on Ap susceptibility would have to be explored (Lennette 1971; Hubalek, Halouzka et al. 1998).

A recent study found two bird species, American robin and veery, as potentially capable of transmitting Ap-ha to naïve *I. scapularis* larvae, suggesting that there may be interspecific variation in avian reservoir competence or avian susceptibility to Ap (Daniels, Battaly et al. 2002). But, the evidence is circumstantial since the larvae could have been infected via co-feeding with an infected nymph that dropped off before the bird was captured. However, our results support the conclusions of Daniels et al. (2002); some birds may be capable of transmitting Ap but there is inter- and intra-specific variation in reservoir competence. Daniels et al. (2002) found that one robin infected 6-35% of feeding ticks. However, only two robins were tested in their study and without a larger sample size it is difficult to draw any conclusions on robin competence for Ap. Birds do not clear the vector of their infection as they have been shown to do for other tick-borne bacteria (Matuschka and Spielman 1992). In fact, in our study, there was a slightly higher prevalence of infection in 0 dpe ticks post-feeding (50%) compared to

pre-feeding (40%); therefore, these birds do appear to allow for some transmission via co-feeding, a point which could explain the results of Daniels et al. (2002).

Since transmission appears so low, a bird's role as a dispersal agent is mainly limited to dispersing infected nymphs. Nymphs could be moved to a new location while feeding and then drop off and molt into adults. Since adults typically feed on deer, and deer are not an epidemiologically important reservoir (Tate, Mead et al. 2005; Reichard, Blouin et al. 2009), this type of dispersal is of little public health importance. Transmission to larvae via co-feeding is also possible, as shown here, but transmission from nymphs to larvae is unlikely since larval and nymphal emergence peaks are separated by 8 weeks in some areas (Gatewood, Liebman et al. 2009) and larvae and nymphs often feed on different locations on a host (pers. obs.). Therefore, though birds appear capable of some transmission via co-feeding and could transport an infected vector to a new location, their role as a dispersal agent for Ap is limited and likely of little public health importance.

There may be factors such as co-infections, and immunosuppressive life-history events, Ap strain differences or host immunocompetence that influence a bird's ability to transmit Ap. For example, the robin in the Daniels et al. (2002) study was concurrently infected with and transmitting Bb, the agent that causes Lyme disease. This type of co-infection could alter the immune status of the bird and make it more susceptible to Ap transmission. Indeed, co-infection of Ap and Bb happens frequently in tick vectors and host reservoirs and has been shown to increase Ap transmission in some cases (Thomas, Anguita et al. 2001; Vaclav, Ficova et al. 2011) though other studies have found interference between the pathogens that limits transmission of both (Levin and Fish 2000;

Levin and Fish 2001). The transmitting robin in this study was wild-caught and therefore could have been exposed to other pathogens before Ap which may have altered its susceptibility. Studies assessing the impact of co-infection on the reservoir competence of wild birds would clarify this possibility.

Furthermore, maintaining immunocompetence can be costly so birds can suppress or enhance immune function seasonally (Nelson and Demas 1996). During an immunosuppressive event, a bird that is typically resistant to infection could become susceptible. Immunosuppression has been shown to occur during migration (Owen and Moore 2008), which is particularly relevant since it might allow an otherwise resistant bird to become a dispersal agent for an infectious disease like Ap.

Previous studies (Alekseev, Dubinina et al. 2001; Skotarczak, Rymaszewska et al. 2006) assessing avian reservoir competence for Ap were conducted in Europe. The predominant Ap strain in Europe is most closely related to our Ap-variant 1 strain rather than the Ap-ha strain used in the current study (Massung, Levin et al. 2007). Ap strains have been shown to vary in host tropism and virulence (Levin and Ross 2004; De La Fuente, Massung et al. 2005; Massung, Mather et al. 2006; Reichard, Blouin et al. 2009). In the present study, we tested the susceptibility of birds to one isolate of Ap-ha (Dawson strain) originating from a tick in Connecticut (Levin and Ross 2004). Though the species tested in this study are not competent reservoirs for the Dawson strain, it is possible that birds could play a role in the transmission of other Ap-ha isolates or of different strains of Ap (ex: Ap-variant 1).

Generally, resistance could be due to an inability of Ap to invade the host (for example, if conditions are preclusive to growth) or to the host's ability to limit the

infection. If the adaptive immune response is not involved, it is likely that an effective innate immune response was mounted, as suggested by the fever exhibited in exposed catbirds. Other aspects of innate immunity, like the actions of white blood cells and the complement cascade could also have been involved. Complement has been shown to be effective at conferring resistance to Bb (Kurtenbach, Sewell et al. 1998) and may be playing a role in avian resistance to Ap as well. Future studies could investigate this possibility.

The challenge of detecting Ap exposure for birds in this study included a lack of bacteremia, transmission, and an inability to detect Ap-specific antibodies. Typically IgM antibodies for Ap are detectable in blood by day seven and predominate over the first two weeks with IgG antibodies becoming detectable 2-3 weeks after exposure (Woldehiwet and Scott 1982; Zeman, Pazdiora et al. 2002; Walder, Falkensammer et al. 2003; Novakova and Vichova 2010). Therefore, at 14 dpe IgM and possibly IgG antibodies would have been detectable and by 28 dpe IgG should have been at maximum detectability. We used a polyclonal anti-wild bird IgG antibody conjugate, which is developed against IgG antibodies, but, given the shared light chains for IgG and IgM, should also give us as low-level detectability for IgM. Thus, our negative results on 14 (including from the one transmitting robin) and 28 dpe suggest that Ap exposure did not elicit an adaptive immune response. This finding broadly indicates that serology cannot be used to detect Ap exposure in wild birds and specifically that our initial tests for exposure to Ap pre-captivity are inconclusive. Given the low level of Ap exposure in the wild (Ogden, Lindsay et al. 2008; Hildebrandt, Franke et al. 2010) and our preferential selection of hatch year birds, it is unlikely that these birds were previously exposed to

Ap. Furthermore, Ap antibodies do not necessarily provide protection even from homologous strains of Ap, so previous exposure would not explain the resistance exhibited in this study.

The challenge of detecting Ap exposure was confounded by the difficulty we experienced in keeping engorged tick with their correct host. Engorged ticks have the potential to be flung when their avian host flaps its wings or shakes its head. During the infestation (0 dpe) period of this study, we found engorged nymphs stuck to the sticky carpet tape surrounding the cages, and in the water pan of birds that were never exposed to nymphs. We also found three infected ticks in the cages of birds exposed only to uninfected ticks. All three birds were either below or bordered by an exposed bird's cage and combined with the above evidence, we find it most likely that these ticks were flung from a nearby exposed individual. We did not find any evidence that the birds themselves were exposed to Ap but given the overall low detectability of Ap-exposure for birds in our study we cannot rule out the possibility. In subsequent rounds, we placed transparent barriers between the cages to reduce contamination and improve overall tick recovery rates. We recommend this strategy or a similar cage-separation method to other studies involving ticks feeding on birds.

We have demonstrated that two species of bird do not acquire infection, nor significant disease from exposure to Ap. They are unlikely to play a role in the natural transmission ecology (although they do seem to allow transmission via co-feeding). However, the lack of disease indicates that exposure to Ap-infected ticks would not preclude or delay a bird from migrating which supports prior assertions that birds may be involved in the dispersal of Ap-infected ticks (Bjoersdorff, Bergstrom et al. 2001;

Skotarczak, Rymaszewska et al. 2006). However, given that birds are unlikely to transmit the infection to feeling larvae, they would only be dispersing infected nymphs, which, given the incompetence of deer as reservoirs for Ap-ha, would not start a new foci of infection.

This study helps to answer some long-standing questions on avian reservoir competence for Ap; however, many more questions have been raised. Additional studies are needed to examine the reservoir competence of other species, especially veerys, in a captive setting and to assess the role that coinfections, immunosuppressive events like migration, and strain differences affect variation in avian susceptibility to Ap. This study was the first step in identifying the role that birds play in the ecology of this pathogen. Further exploration into this topic will give us a more robust understanding of Ap transmission and dispersal dynamics.

SUPPLEMENTARY MATERIALS

Table 1. Infection prevalence for each individual on each round of tick attachment. Shown as number of Ap-positive ticks/total number of ticks tested (percent infected).

		Exposure	**Transmission during xenodiagnostic event at**			
Bird	**Treatment**	**0 dpe**	**7 dpe**	**14 dpe**	**42 dpe**	**77 dpe**
B28	CatExp	1/4 (25)	0/19 (0)			
Br61	CatExp	3/6 (50)	0/16 (0)	0/12 (0)	0/25 (0)	0/12 (0)
Br63	CatExp	2/5 (40)	0/12 (0)			
Br66	CatExp	1/2 (50)	0/11 (0)	0/24 (0)	0/18 (0)	
G54	CatExp	3/3 (100)	0/14 (0)	0/2 (0)		
R110	CatExp	4/8 (50)	0/15 (0)	0/7 (0)	0/38 (0)	0/10 (0)
R20	CatExp	2/3 (67)	0/13 (0)			
W16	CatExp	4/5 (80)	0/20 (0)	0/6 (0)	0/16 (0)	0/11 (0)
Y42	CatExp	2/9 (22)	0/18 (0)			
Y43	CatExp	6/10 (60)	0/22 (0)	0/19 (0)	0/24 (0)	0/18 (0)
14G	CatTic		0/19 (0)			
Br67	CatTic	0/2 (0)	0/10 (0)			
Y82	CatTic	1/4 (25)	0/12 (0)			
B1	MouInf	7/8 (88)	17/18 (94)	21/21 (100)	0/11 (0)	0/7 (0)
B2	MouInf	3/7 (43)	2/2 (100)	16/16 (100)	11/13 (85)	0/12 (0)
B3	MouInf	4/6 (67)	16/18 (89)	19/22 (86)	11/12 (92)	0/12 (0)
B4	MouInf	3/6 (50)	11/12 (92)	6/13 (46)	4/5 (80)	0/7 (0)
G71	RobCon		0/7 (0)			
G75	RobCon		0/12 (0)			
G78	RobCon		0/12 (0)			
P54	RobCon		0/12 (0)			
P58	RobCon		0/12 (0)			
P60	RobCon		0/11 (0)			
R6	RobCon		0/10 (0)			
W82	RobCon		0/10 (0)			
W83	RobCon		0/6 (0)			
W87	RobCon		0/11 (0)			
G77	RobExp	2/2 (100)	0/7 (0)			
G79	RobExp	1/3 (33)	0/7 (0)	0/25 (0)	0/7 (0)	0/12 (0)
P51	RobExp	4/5 (80)	0/17 (0)			
P52	RobExp	1/2 (50)	2/13 (15)			
P53	RobExp	2/4 (50)	0/19 (0)	0/47 (0)	0/16 (0)	0/12 (0)
P55	RobExp	1/3 (33)	0/5 (0)	0/27 (0)	0/10 (0)	0/9 (0)
P56	RobExp	3/7 (42)	0/9 (0)			
R8	RobExp	0/2 (0)	0/7 (0)	0/27 (0)	0/12 (0)	0/1 (0)
R9	RobExp	2/4 (50)	0/7 (0)			
W86	RobExp	2/5 (40)	0/7 (0)	0/25 (0)	0/35 (0)	

Table 1. (cont'd)

		Exposure	Transmission during xenodiagnostic event at			
Bird	**Treatment**	**0 dpe**	**7 dpe**	**14 dpe**	**42 dpe**	**77 dpe**
G73	RobTic		0/12 (0)		0/2 (0)	
G74	RobTic		0/12 (0)		0/2 (0)	
G80	RobTic	0/1 (0)	0/3 (0)	0/7 (0)	0/2 (0)	
P57	RobTic		0/13 (0)			
R4	RobTic	1/8 (13)	0/13 (0)			
R5	RobTic	1/5 (20)	0/1 (0)	0/15 (0)		
R7	RobTic		0/3 (0)			
W81	RobTic		0/12 (0)			
W84	RobTic		0/16 (0)		0/1 (0)	
W85	RobTic	0/4 (0)	0/8 (0)			

Figure 6. Average catbird temperatures (± 1 standard error) over time

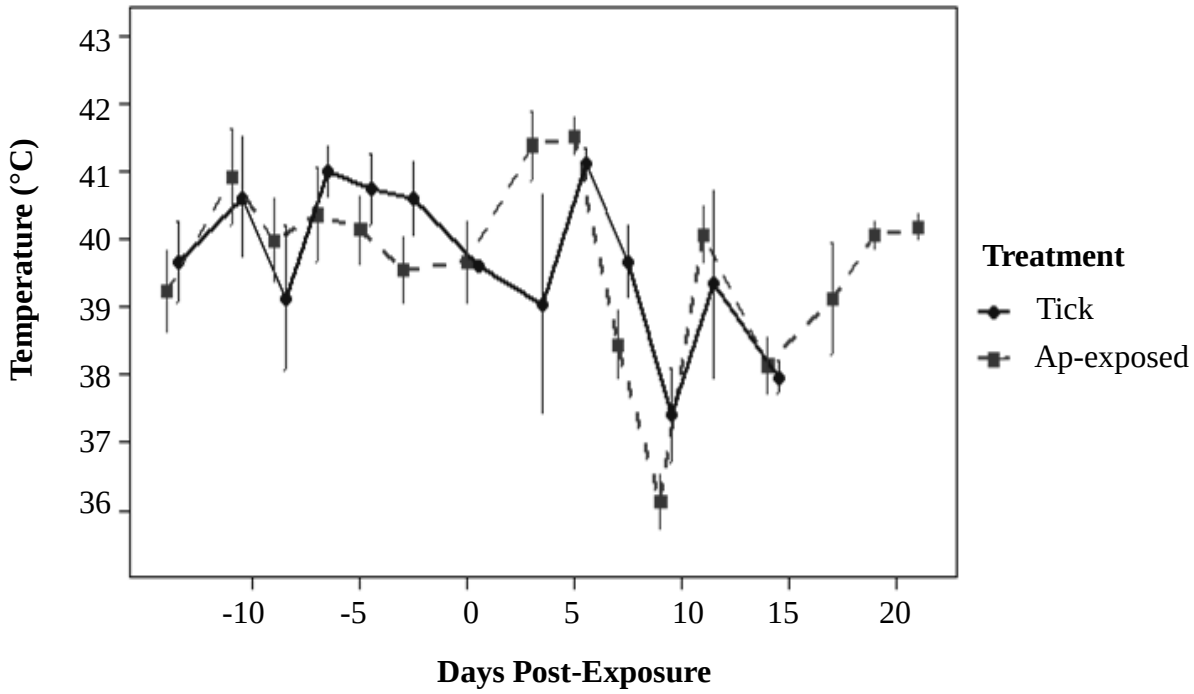


Figure 7. Average American robin temperatures (± 1 standard error) over time

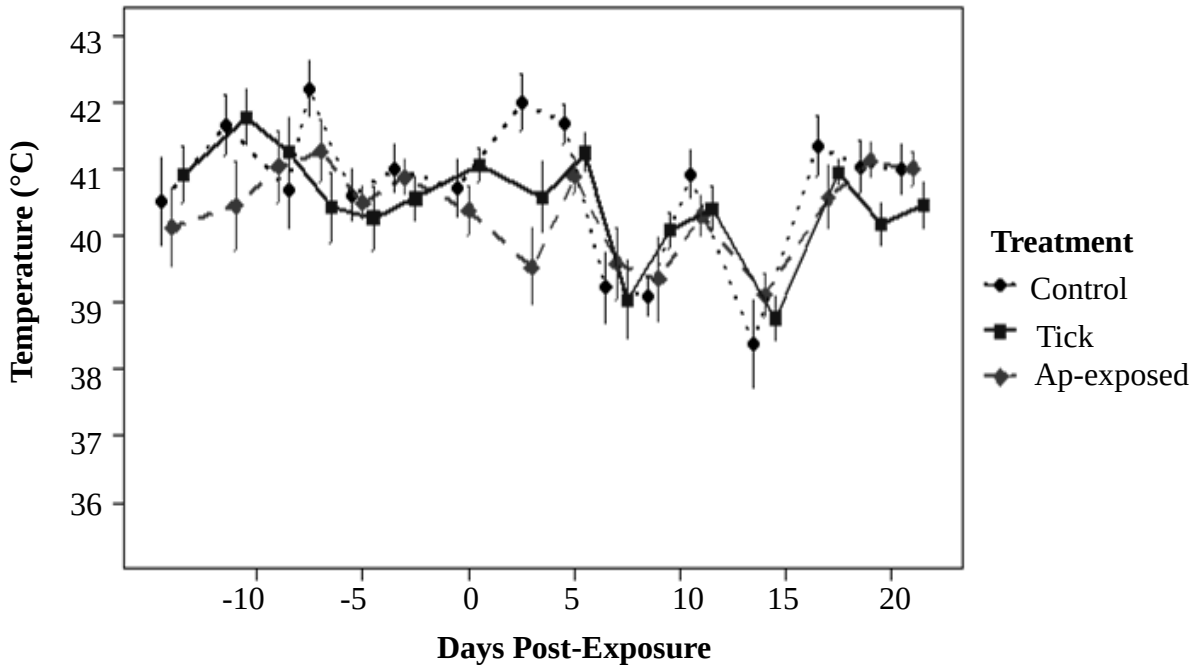


Figure 8. Average gray catbird relative mass change (± 1 standard error) over time

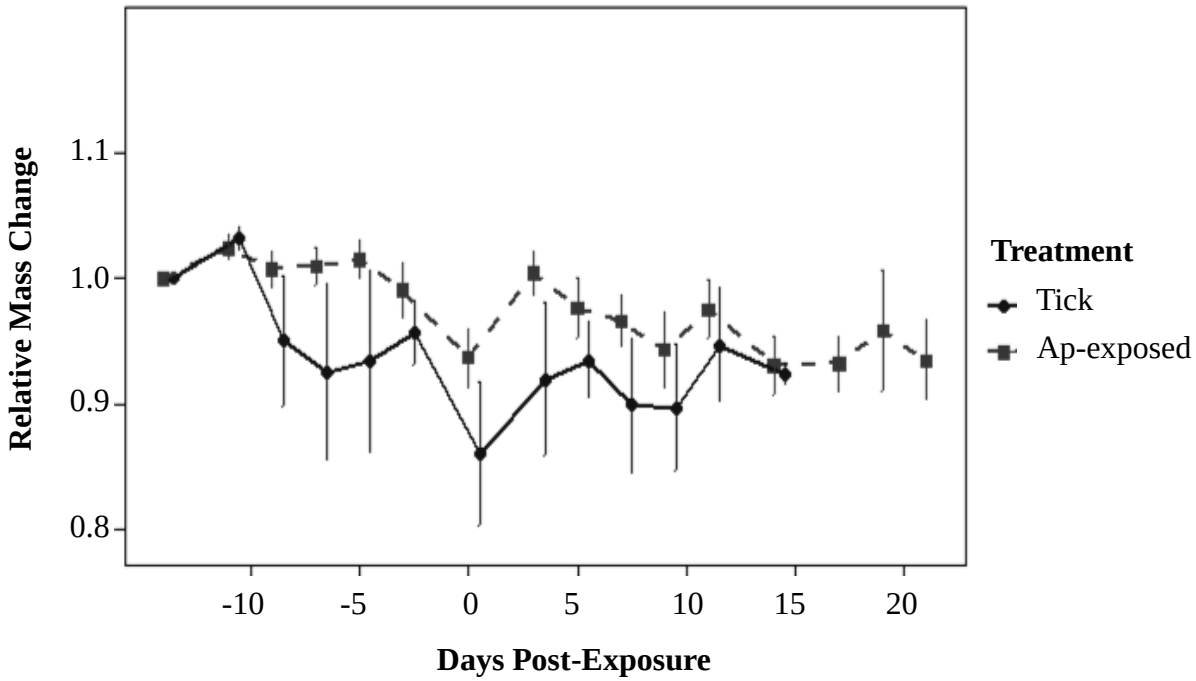
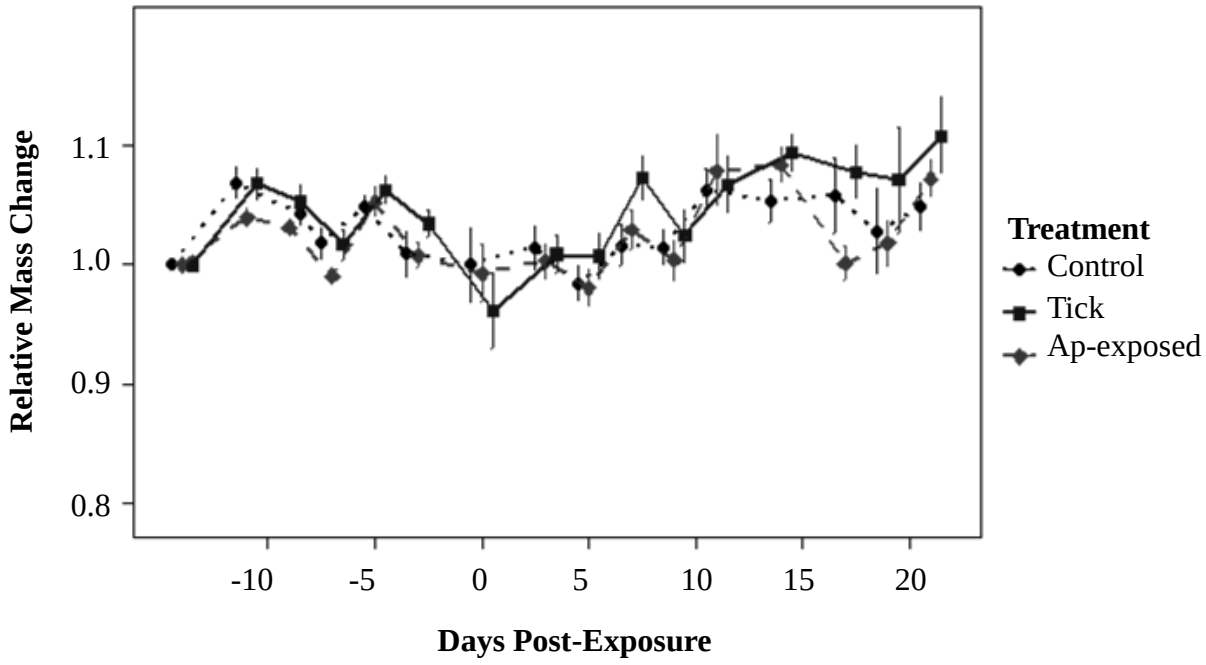


Figure 9. Average American robin relative mass change (± 1 standard error) over time



Supplement 1. RNA preservative recipe

Prepare or obtain the following stock solutions and reagents:

0.5 M EDTA (Ethylenediaminetetraacetic acid) disodium, dehydrate (18.61 g/100 ml, pH to 8.0 with NaOH while stirring)
1M Sodium Citrate
Trisodium salt, dihydrate (29.4 g/100 ml, stir to dissolve)
Ammonium Sulfate, powdered
Sterile water.

In a beaker, combine 40 ml 0.5 M EDTA, 25 ml 1M Sodium Citrate, 700 gm Ammonium Sulfate and 935 ml of sterile distilled water, stir on a hot plate stirrer on low heat until the Ammonium Sulfate is completely dissolved. Allow to cool, adjust the pH of the solution to pH5.2 using 1M H_2SO_4 (sulfuric acid). Transfer to a screw top bottle and store either at room temperature or refrigerated.

Supplement 2. Recipe for bird food

Cricket Mash

1) Place 4 cups frozen blueberries into food processor
2) Add 1.5 cups frozen crickets
3) Add 4 cups cottage cheese
4) Set to mix
5) While mixing, pour 2 boxes of nutty nuggets (or some other generic brand of Grape Nuts cereal) into a large bin.
6) When CM is done mixing, pour it over the nutty nuggets.
7) Mix the ingredients thoroughly so as to make sure that there are no pockets of hard nuggets
8) Place back in refrigerator to absorb moisture overnight
9) Separate into 3-4 plastic containers and keep in fridge for no more than 3 days or in freezer.

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