Potential Downstream Immunological Effects of Evolved Disease Tolerance in House Finches

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Academic Abstract

Emerging infectious diseases can exert strong selection on hosts to evolve resistance or tolerance to infection. However, it remains unknown whether the evolution of specific defense strategies against a novel pathogen influences host immune phenotypes more broadly, potentially affecting their ability to respond to other pathogens. In 1994 the bacterial pathogen, *Mycoplasma gallisepticum* (MG) jumped from poultry into house finches, causing severe conjunctivitis and reducing host survival. MG then spread across the continental United States, exerting strong selection on host populations and creating geographic variation in the degree of population co-evolutionary history with the pathogen. Prior work found that populations of house finches with longer histories of MG endemism have evolved tolerance and resistance to MG, and this evolution is associated with several immunological differences including reductions in pro-inflammatory immune responses. However, it remains unknown whether these immunological changes are limited to MG-specific defenses or whether broader immune responses differ between populations with distinct coevolutionary histories with MG. To examine possible effects of the evolution of host responses to MG, we used five immune assays to challenge house finches from four populations, ranging from no history of MG endemism to 20+ years of MG endemism. When challenged with phytohemagglutinin (PHA), populations differed significantly in the strength of wing web swelling, with populations with longer MG exposure (and thus the highest MG tolerance) on average exhibiting the weakest swelling response when mass differences were controlled for. However, detected population differences in wing web swelling were small, and population differences were absent for responses to four other immune assays that spanned components of the innate and adaptive immune system. Future work should examine whether the local inflammation that underlies swelling responses to PHA shares common immunological mechanisms with local inflammatory responses to MG, which may explain why populations with evolved tolerance to MG show slightly lower swelling responses in response to PHA. Overall, these results suggest that the evolution of MG tolerance may have minor downstream

consequences for responses to certain antigens, with the potential to influence a host's ability to respond to novel pathogen challenges, but most components of the host immune system appear largely unaffected.

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General Audience Abstract

Emerging infectious diseases can have devasting effects on new host species. To reduce the cost of these pathogens, host species can evolve ways to eliminate infection (resistance) or reduce damage during infection (tolerance), which is often caused by the host's immune system itself. As populations evolve these disease strategies, it is likely that other aspects of the immune system will also be affected, potentially compromising the ability of hosts to respond to pathogens other than the ones they evolved defenses against. We examined what sort of trade-offs might arise as house finches evolved resistance and tolerance to a new deadly pathogen, *Mycoplasma gallisepticum* (MG). House finch populations in the mid-Atlantic were first exposed to the disease in 1994, and as the disease spread across the continental United States, different populations have been exposed for different periods of time. This created a gradient in whether certain populations have had long enough time with MG to evolve disease strategies. Populations that have been exposed to MG for longer appear to have evolved both resistance and tolerance, and tolerant populations show lower levels of inflammatory immune markers that can be associated with self-damage. Using house finches from four different populations (ranging from 25 years of exposure history to zero years of MG exposure history), we tested a variety of immune system components to examine what areas of the immune system might have been broadly affected by the evolution of resistance and tolerance. We hypothesized that birds from populations with evolved MG tolerance would also have a reduced inflammation response when stimulated with substances that mimic infection by something other than MG. Only one assay supported this hypothesis. Birds from populations that had been exposed to MG for a longer period of time (and thus had evolved MG tolerance) had a reduced swelling response following injection with a plant protein called phytohemagglutinin. However, there were no population differences observed with the other four assays, suggesting that evolving defenses against MG did not result in widespread immunological effects. This suggests that the evolution

of host defenses against an emerging pathogen may not compromise that host's ability to respond effectively to other types of pathogens that they encounter in nature.

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Chapter 1 – Introduction

Organisms are not able to have it all. Maximizing all components of fitness (aka, living a long time and having many offspring every year) would be ideal but is unachievable in the natural world due to trade-offs (Stearns, 1992). Within an organism's lifespan and throughout generations, trade-offs will occur in response to constraints and limited resources. Trade-offs can arise in which organisms must allocate limited resources among competing components of fitness. These physiological trade-offs can occur between traits that contribute to reproductive success versus survival, the two key components of individual fitness (Stearns, 1992). For example, female deer (*Cervus elaphus*) prioritize their current reproductive success over their own survival when they use body fat to feed offspring through milk production, rather than reserving fat stores for themselves to use for survival over winter (Clutton-Brock, Guinness, & Albon, 1982). Physiological trade-offs can also occur with respect to the immune system, which is often used as a proxy for individual investment in "survival." For example, investment in reproduction by an organism is often associated with reductions in the strength of their immune responses or increases in rates of parasitism, suggesting a resource based trade-off between reproduction and the ability to mount immune responses (Gustafsson, Nordling, Andersson, Sheldon, & Qvarnström, 1994; Schwenke, Lazzaro, & Wolfner, 2016). Similarly, mounting an immune response can negatively impact growth rates (Saino, Calza, & Møller, 1998). These studies together suggest that mounting immune responses can be physiologically costly.

In addition to physiological trade-offs, evolutionary trade-offs occur across generations. Evolutionary trade-offs are often seen when a population faces a selection pressure and in response evolves a change in one trait that then has downstream effects on another trait. Evolutionary trade-offs are based on the concept that the evolution of one trait will often carry consequences for other traits, frequently causing an effect on overall fitness. Evolutionary trade-offs have been long studied in the context of life-history trade-offs, providing answers for why animals cannot simultaneously evolve to maximize every component of fitness. For example, the evolution of large offspring is often associated with a longer time

to reach maturity (Stearns, 1992). Through exploring evolutionary trade-offs, we have been able to better understand the constraints that arise when populations evolve in response to various selection pressures. These constraints can arise for traits that are not life-history traits, but instead depend on common molecular mechanisms. For example, in garter snake (*Thamnophis sirtalis*) populations that evolved to eat toxic prey through modification of a sodium channel, it was found that locomotion in populations that had evolved the most complete resistance to the toxin was decreased (Hague et al., 2018). Because locomotion also uses sodium channels, a clear trade-off was observed among populations that had evolved mechanisms to withstand toxic prey, inadvertently affecting their ability to move.

Evolutionary trade-offs have been examined in response to many selection pressures, including the presence of pathogens. In the context of life-history theory, these studies often examine the fitness costs that occur in response to the evolution of a disease related trait such as resistance to a parasite or pathogen. Resistance is generally defined as a host reducing or eliminating pathogen load from the infected tissue(s) (Råberg, Sim, & Read, 2007). Because resistance to a pathogen can be costly, the evolution of resistance can result in trade-offs for other traits. For example, when fruit flies (*Drosophila melanogaster*) were selected to be resistant to a bacterium (*Pseudomonas aeruginosa*), the selected flies had reduced longevity and produced less viable eggs (Ye, Chenoweth, & McGraw, 2009). Aside from these classic life-history trade-offs in response to pathogens, much work has also been done examining how evolving resistance to one parasite or pathogen affects other immune traits or the ability of the host to defend itself from other pathogens. For example, a study conducted on fruit flies looked at how evolving resistance to a parasitoid wasp affected resistance to the other parasitoid species. This study found that in some cases, evolving resistance to one species of wasp could help improve defense against another species; however, this evolution did not provide resistance to all other parasitoid wasp species (Fellowes, Kraaijeveld, & Godfray, 1999). This work shows that evolving resistance can have effects on responses to some, but not all other parasites and pathogens, suggesting that the evolutionary costs (or benefits) of host responses such as resistance may vary. Another artificial selection experiment produced

lines of chickens that were selected to produce either high or low antibody titers when exposed to sheep red blood cells. The high antibody (HA) and low antibody (LA) lines were then exposed to a range of disease challenges to see how selection for high or low antibody responses to one antigen affects selection on other immune traits. When challenged with a variety of pathogens, the HA line was more resistant to infections with the bacterial pathogen *Mycoplasma gallisepticum* and the protozoal parasite *Eimeria necatrix*, but less resistant to the bacterial pathogens *Escherichia coli* and *Staphylococcus aureus* (Gross, Siegel, Hall, Domermuth, & DuBoise, 1980). This work again highlights how evolving to be more responsive to one particular antigen may lead to downstream effects for other components of the immune system. It also suggests that effects on the immune system might not be all-encompassing, and the direction and intensity of the effect might be highly variable in response to diverse pathogens.

While ample previous work has examined the costs of resistance, much less work has examined what evolutionary trade-offs might arise through the evolution of host tolerance. Tolerance and resistance are two different disease strategies that aim to reduce the fitness effects of a pathogen in distinct ways. Tolerance is similar to resistance in that both strategies allow for a decrease in the total fitness cost of infection, but while resistance reduces the fitness cost through a reduction in pathogen load, tolerance reduces fitness cost through mediating the damage caused by the pathogen. With a reduction in pathogen load, infection severity is decreased, which allows for less damage to occur to the host and thus for fewer resources to be needed for repair. Generally, the mechanisms that are thought to evolve for disease resistance include components of the adaptive immune response, including increases in specific antibody or T-cell mediated killing/identification/phagocytosis to reduce or eliminate pathogen load (Bonneaud, Pérez-Tris, Federici, Chastel, & Sorci, 2006). Tolerance also reduces the fitness costs caused by a pathogen, but not by directly reducing pathogen load (Råberg et al., 2007). With tolerance, it is thought that pathogen load generally remains the same, but the mechanisms that can cause damage during infection (in some cases, the host's own immune response) are reduced, allowing a host to maintain fitness while still harboring a given pathogen burden (Medzhitov, Schneider, & Soares, 2012). While the

mechanisms of tolerance are unknown for most host-pathogen systems, in many cases the mechanisms of preventing damage, and thus increasing tolerance, may be more general (i.e., related to innate, non-specific immunity or tissue repair) than those associated with resistance. Because the mechanisms for resistance and tolerance are often through different components of the immune system, the evolutionary costs or benefits of tolerance may be predicted to be different than those imposed by resistance. However, to date, no studies have examined how the evolution of host tolerance alters other types of immune responses in positive or negative ways.

The work to date on evolutionary trade-offs that result from evolving defenses to particular antigens or parasites has largely been done in artificial settings. Studies of natural systems are an important complement to artificial selection studies because they provide critical insight into the downstream evolutionary effects that result in wild populations subject to diverse selection pressures. My work leverages a naturally-occurring host-pathogen system where the evolution of host resistance and tolerance have been documented, to examine specifically whether the evolution of host responses to an infectious disease results in detectable changes to the host's immune response to other antigens. House finches (*Haemorhous mexicanus*) were first documented with a unique strain of the poultry bacterium *Mycoplasma gallisepticum* (hereafter referred to as MG) in 1994 (Ley, Berkhoff, & McLaren, 1996), and the pathogen has since spread throughout the continental United States. Populations that have been exposed to MG for longer periods of time have evolved both resistance and tolerance to this pathogen, allowing us to examine how this evolution has affected the immune system more broadly. Because prior work suggests that dampened inflammation is a potential mechanism of tolerance in this system (Adelman, Kirkpatrick, Grodio, & Hawley, 2013), we hypothesized that the evolutionary changes associated with tolerance in this system may have influenced the way that the innate immune system responds to a diversity of antigens. Thus, we focus heavily on assays of innate immunity (Bonneaud, Balenger, Zhang, Edwards, & Hill, 2012), but given prior evidence that responses to MG relate to antibody responses to sheep red blood cells in chickens (Gross et al., 1980), we also examine potential

downstream effects of MG resistance on antibody responses to sheep red blood cells. Overall, my work uses a variety of immune challenges to examine the possible evolutionary effects that have arisen in a wild host that has experienced natural selection pressures from disease. By doing so, we can understand how host evolution to combat one pathogen may ultimately influence the ability of that host to combat other pathogens with potential fitness effects. Thus, the results of our work have important implications for understanding both ecological (e.g., coinfection) and evolutionary (e.g., co-evolution) dynamics between hosts and the variety of pathogens that place continual selection on them.

Chapter 2 - Potential Downstream Immunological Effects of Evolved Disease Tolerance in House Finches

Introduction

Infectious diseases constantly emerge within new host populations. These novel pathogens often spread rapidly and can cause significant host mortality, placing heavy selection pressure on host populations to adapt. There are two primary strategies that hosts can evolve to reduce the fitness toll of pathogen infection. Resistance is defined as the reduction or elimination of pathogen load, and tolerance is defined as minimizing per-pathogen impacts on host fitness. Recent work suggests that host populations can often rapidly evolve one or both strategies to combat emerging infectious diseases. White Nose Syndrome, caused by the fungus *Pseudogymnoascuc destructans*, resulted in sharp and drastic declines in numerous bat populations, but some populations are stabilizing and harbor lower pathogen intensities, which suggests that some species have begun to evolve resistance to the fungal pathogen (Langwig et al., 2017). It has also been suggested that frogs exposed to *Batrachochytrium dendrobatidis* have begun to evolve mechanisms to tolerate this emerging and deadly fungus (Savage & Zamudio, 2016). Evolving strategies such as resistance or tolerance can help to mitigate population declines and reduce the fitness costs of infectious disease on hosts. However, it remains unknown whether evolutionary trade-offs result as free-living hosts evolve the mechanisms for resistance or tolerance to emerging diseases.

Evolutionary trade-offs have been heavily studied using artificial selection experiments and in the context of disease resistance. For instance, when turkeys were artificially selected for high weight gain and then challenged with a bacterial pathogen, the high weight strain had reduced survival in response to infection relative to the low weight selected-strain (Nestor, Noble, Zhu, & Moritsu, 1996). These trade-offs have also been observed in studies of artificial selection for a disease strategy. When fruit flies (*Drosophila melanogaster*) were selected for resistance against the bacterium *Pseudomonas aeruginosa*,

fruit flies bred to be resistant to the pathogen had decreased longevity and lower egg viability (Ye et al., 2009).

While these studies suggest that a suite of traits can show evolutionary trade-offs with pathogen defense, a host's immune system may be particularly likely to be affected more broadly by the evolution of disease strategies against a given pathogen. For example, when chickens were selected for either a high or low antibody titer response to sheep erythrocytes, the high antibody line of birds had increased resistance to the bacterial pathogen *Mycoplasma gallisepticum* and the protozoal parasite *Eimeria necatrix*, but reduced resistance to the bacterial pathogens *Escherichia coli* and *Staphylococcus aureus*, relative to low antibody birds (Gross et al., 1980). In some systems, genetic correlations have been detected between components of the immune response, suggesting that selection for one immune trait may be particularly likely to influence another (Cotter, Kruuk, & Wilson, 2004). Together, these studies suggest that evolving tolerance or resistance to a pathogen may, in some cases, lead to downstream trade-offs that affect other components of the immune system in either negative or positive ways. However, most of the work to date on evolutionary trade-offs associated with immunity or disease resistance has been done via artificial selection rather than within systems where a disease strategy arose through natural selection in response to an emerging pathogen. One study of multiple European rabbit (*Oryctolagus cuniculus*) populations began to examine the possible downstream immunological effects that arise when populations evolve resistance to a pathogen via natural selection (Alves et al., 2019). Populations of rabbits with evolved resistance to myxoma virus had altered antiviral gene sequences that also appeared to confer increased antiviral activity against several viruses unrelated to myxoma. Thus, the authors hypothesize that the evolution of resistance to myxoma virus may also confer increased resistance to other viruses in rabbits.

House finches and their bacterial pathogen *Mycoplasma gallisepticum* (hereafter MG) provide an exciting opportunity to similarly examine how the evolution of host responses to an emerging disease can influence the host immune system more broadly. Originally a pathogen primarily of poultry, MG spilled over into house finch (*Haemorhous mexicanus*) populations in the mid 1990s (Ley et al., 1996). MG

infection in house finches is characterized by severe conjunctivitis (inflammation of the conjunctiva) (Dhondt et al., 2005) that indirectly results in high rates of mortality within wild populations (Faustino et al., 2004). When MG first spilled over into house finch populations, it caused sharp population declines of up to 60% in free-living affected populations (Hochachka & Dhondt, 2000).

The noticeable clinical signs of this disease allowed for the spread of MG across the country to be readily characterized (Dhondt et al., 2005). MG was first documented in Virginia and moved rapidly through the eastern, introduced range of the house finch, and then more slowly across the top of the continental United States, creating populations that had been exposed to MG for distinct amounts of time (Staley, Bonneaud, McGraw, Vleck, & Hill, 2017). This allows for space-for-time studies that can use population differences in coevolutionary history and disease response to infer pathogen-mediated evolution. Populations that have been exposed to MG for longer appear to have evolved resistance to MG, as evidenced by reduced pathogen loads during infection (Bonneaud et al., 2011). However, other studies have found that tolerance has evolved, as evidenced by reductions in per-pathogen conjunctivitis severity and mass loss in populations with a longer exposure history to MG (Adelman et al., 2013). Recent work suggests that both strategies may have evolved simultaneously in house finch populations (Bonneaud et al., 2019). What is not yet known is whether the evolved resistance and/or tolerance to MG resulted in downstream evolutionary effects for the host immune system more broadly. Our work begins to examine this possibility in house finches captured from four different populations that vary in their degree of evolved tolerance and resistance to MG (Table 1).

Table 1. House finch p**opulations** used in this study, **and** their e**xposure** h**istories** with ***Mycoplasma gallisepticum*** **(MG).**

Populations	**Years Exposed to MG**
Virginia	25+
Iowa	20+ years
Arizona	5-10
Hawaii	No prior exposure

The degree to which the evolution of resistance or tolerance to MG influences the ability of the host immune response to respond to other pathogens will likely depend on the specific immune mechanisms associated with resistance and tolerance in this system, as well as the specificity of any resulting immune

trade-offs. Intriguingly, house finch populations with evolved tolerance to MG also show reduced expression of pro-inflammatory cytokine responses early in infection (Adelman et al., 2013), suggesting that one mechanism of tolerance in this system may be the suppression of innate inflammatory responses that are associated with virulence in this system (Vinkler, Leon, Kirkpatrick, Dalloul, & Hawley, 2018). Thus, we predicted that populations with evolved tolerance to MG might also show reduced inflammatory responses to other antigens. The inflammatory response can be stimulated through recognition of pathogen associated molecular patterns by Toll-like receptors (TLR). Thus, the degree to which evolutionary trade-offs in inflammation result from the evolution of tolerance to MG may be a function of whether antigens are detected via the same TLR(s) as MG. Because MG is recognized largely through TLR-2 (Majumder, Zappulla, & Silbart, 2014), we selected one antigen (fibroblast-stimulating lipoprotein-1 or FSL-1) that also stimulates TLR-2 (Ahmad, Shihab, Jasem, & Behbehani, 2014) to determine if any detected reductions in the degree of inflammatory response to other antigens are TLR-specific. We also used lipopolysaccharide (LPS), which is recognized by TLR-4, as an antigen that uses a distinct TLR pathway from MG to test the possibility of a non-TLR specific reduction in inflammatory responses associated with the evolution of MG tolerance.

One common way to measure the strength of the systemic inflammatory response in vertebrates, which appears to be altered in populations with evolved MG tolerance (Adelman et al., 2013), is by examining the acute phase response (APR). The APR is characterized by systemic changes such as fever, anorexia (which leads to mass loss), and lethargy due to sickness behaviors (Kushner, 1982). Because APR expression is stimulated by pro-inflammatory cytokine expression (Baumann & Gauldie, 1994), measuring aspects of the APR provides one way to examine the innate inflammatory response as a possible area of the immune system where trade-offs in response to MG-evolved tolerance could occur.

Another way to measure the host inflammatory response is using the degree of inflammation produced by a localized injection. Phytohemagglutinin (PHA) is a lectin derived from red kidney beans, and when injected into the wing patagium, causes non-specific swelling through the recruitment of leukocytes from

the blood stream to the injection site (Vinkler, Bainová, & Albrecht, 2010). By measuring the swelling response post injection, the immune response to a foreign antigen can be captured, with a larger swelling response suggesting a more robust inflammatory response. Through using these techniques, we aimed to understand whether and where trade-offs associated with the strength of inflammation have arisen as house finch populations have evolved tolerance to MG. We also examined one immune metric potentially associated with the evolution of resistance to MG in house finches: the strength of antibody responses to sheep red blood cell (SRBC) injection. Because prior work found that chickens selected for high responses to SRBCs showed stronger resistance to poultry strains of MG (Gross et al., 1980), we asked whether the natural evolution of resistance to MG in house finches is associated with the ability to produce stronger antibody responses to SRBCs.

Our work used house finches captured from four different populations that vary in their degree of coevolutionary history with MG (Table 1), and thus their evolved resistance and tolerance, to address whether the evolution of host responses resulted in trade-offs or benefits in the host's ability to respond to other antigens. We assess these potential trade-offs or benefits using a series of immune challenges that span components of the innate and adaptive immune system. We hypothesized that house finches from populations that show evolved tolerance to MG would show reduced APR expression when injected with antigens such as LPS and FSL-1, with the strongest reductions detected in response to FSL-1, which shares the same TLR as MG. We also hypothesized that house finches from populations with evolved tolerance would have a reduced swelling response when injected with PHA. Finally, we hypothesized that populations with evolved resistance to MG would show stronger antibody responses to SRBC injection.

Methods

Bird Capture and Housing

We trapped and retained house finches from four populations that vary in their degree of co-evolutionary history with MG (Table 1). Using mesh wire traps and mist nets, we captured mixed sex, hatch-year house finches between June-September 2018 in Tempe, Arizona (N=28), Dillingham Air Field, Hawaii (N=32), Ames, Iowa (N=32), and Blacksburg, Virginia (N=43). All birds were aged via plumage. Following capture, birds were transferred via vehicle or airplane to either Virginia Tech (N=88) or Iowa State University (N=47) for an experimental inoculation study. For that study, a subset of birds naive to MG at capture were inoculated with varying doses of the pathogen in October 2018, and host responses to infection were quantified over four weeks post-inoculation (Henschen et al., in prep). At the end of the experiment (November 2018), a subset of birds housed at Iowa State University for the infection experiment were transferred in flock cages via state vehicle to Virginia Tech. Upon arrival, birds that still showed conjunctival pathology received 1g/1L dose of Tylan for 21 days to clear any remaining infection from the prior inoculation experiment. The experiments presented here were conducted approximately three months after any experimental bird had visible pathology. Experimental infection history of birds in our experiment were evenly distributed among our treatment groups and were controlled for in statistical analyses. Because several birds from each population (n=12-20 total per population) served as uninfected controls or were not used in any way in the prior experimental inoculation study, we were able to ascertain that prior MG infection did not notably alter responses to the antigens studied here.

For the purposes of this study, finches were double housed in wire mesh cages (76 cm x 46 cm x 46 cm) in a biosafety level 1 animal facility. Birds were provided ad libitum water and food (Daily Maintenance Diet, Roudybush Inc., Woodland, CA) and maintained on a 12-hour light:dark cycle and at a 21-22°C room temperature. To minimize effects of coccidiosis, a common intestinal disease, finches received Endocox (2.5% toltrazuril) at 1.32g/L, twice a month, alongside twice weekly probiotics treatments in their water. Animal capture was approved by federal (USFWS MB158404-1 and MB82600B-4) and state permits (VDGIF 61440, SP624698, WL19-16, SC1133) and procedures for animal care and use were approved by Virginia Tech's and Iowa State's Institutional Animal Care and Use Committees.

Experimental Design and Timeline

To examine the possibility of population differences in immune responses including local inflammation, acute phase responses, and antibody responses, house finches from four populations that vary in their degree of co-evolutionary history to MG (Table 1) were injected *in vivo* with up to four foreign antigens at different times (Table 2). First, all individuals received a subcutaneous injection with PHA (March 2019) to measure localized inflammation, and then a subset of individuals from each population (Table 3) were injected with either LPS (April 2019) or FSL-1 (May 2019) to quantify components of the acute phase response. In order to minimize potential effects of host responses to one antigen on the other, we waited one month after PHA injection before performing other *in vivo* assays, and we did not inject any individuals with both LPS and FSL-1. However, because some individuals served as controls for LPS or FSL-1 injections and thus had blood samples taken at both time periods, antigen injections were given a minimum of 2 weeks apart. Finally, approximately two weeks later (May 2019), all individuals were injected intramuscularly with sheep red blood cells (SRBCs) to measure antibody responses.

Table 2. Experimental timeline with the order of immune assays as well as whether all or a subset of birds from each population were used for a given assay.

Experimental Day	Date	Experiment	Birds Used
Day 0-4	March 20-23 2019	PHA Injections and measurements	All
Day 29-28	April 18-23 2019	LPS Injections and measurements	Subset
Day 40-42	May 2-4 2019	FSL-1 Injections and measurements	Subset
Day 54-55	May 16-17 2019	Baseline blood collection and SRBC injections	Subset
Day 61-62	May 23-24 2019	Post SRBC injection blood collection	Subset

Table 3. The number of house finches from each population used for each immune assay. Numbers of controls in assays that required controls are presented in parentheses. Sex ratios were approximately 50:50 within population for every immune assay. The asterisk signifies assays where fever data were collected for a subset of birds due to the need to take temperatures within three minutes of entering the room (thus 1-6 birds from each population and treatment combination were injected and had mass data collected, but did not have temperature taken).

Populations	Years Exposed to MG	LPS*	FSL-1*	PHA	SRBC
Hawaii	0 Years	17 (6)	18 (6)	32	32
Arizona	0-5 Years	19 (8)	19 (7)	28	28
Iowa	20+ Years	19 (7)	20 (7)	32	32
Virginia	25 years	19 (7)	20 (7)	39	39
Total		**76**	**77**	**131**	**131**

Local inflammation

To measure local inflammation, we used the PHA assay which measures leukocyte recruitment to the wing patagium following injection of a mitogen (Bílková, Vinklerová, & Vinkler, 2015; Vinkler et al., 2010). First, all birds were weighed using a digital scale (accuracy 0.1 g), and initial skin thickness of the center of the left wing patagium (wing web) was measured three times (to the nearest 0.01 mm) using calipers (Mitutoyo #7301, accuracy 0.01mm). Phytohemagglutinin-L (PHA-L; Sigma-Aldrich product #L2769) was reconstituted with sterilized Dulbecco's PBS (DPBS; Sigma-Aldrich product #D5652). All birds were then injected with a dose of 0.1 mg of PHA-L in 20uL of DPBS subcutaneously into the left

wing patagium. Twenty-four hours (± 30 minutes) post-injection, three left wing patagium thickness measurements were taken again. For each time point, we used the average of three thickness measurements in analyses, comparing post-injection average measurements to pre-injection average measurements.

Acute phase responses

The acute phase response to LPS (derived from *Escherichia coli*; Sigma-Aldrich product #L2880) and FSL-1 (AbCam product #ab144863) was assessed for a subset of birds from all four populations (Table 3). Birds randomly assigned to LPS treatment within each population were weighed and then injected subcutaneously near the keel with 2 mg/kg (0.002 mg/g) LPS dissolved in 1x phosphate buffered saline (PBS). Control birds were not injected as prior work has shown that injection with saline doesn't induce APR (Owen-Ashley, Turner, Hahn, & Wingfield, 2006). For a subset of birds from each population, baseline cloacal temperatures were collected within 3 minutes of entering the captive room. Temperatures were taken with thermologgers and thermocouplers (Omega product #HH801B and #5SC-TT-T-30-36) inserted into the cloaca using petroleum jelly as a lubricant. Six hours post-injection, cloacal temperature within three minutes of room entry and mass were re-collected. For the FSL-1 trials, a similar procedure was followed where after a subset of birds had their cloacal temperature taken, they were then weighed and injected subcutaneously near the keel with 0.1 mg/kg FSL-1 reconstituted into 1X PBS. Six hours post-injection, temperature and mass were taken again.

Antibody responses

The possibility of population differences in antibody production in response to a novel antigen was assessed using SRBC injection. All birds from each population were weighed, whole blood (approximately 50uL) was collected via wing vein puncture into heparinized microcapillary tubes, and then birds were injected intramuscularly into the breast muscle with 40% sheep erythrocytes (SRBCs) diluted with sterilize PBS in a total volume of 50uL. Post-injection blood samples were collected after

seven days. Whole blood from both time points was kept at 4°C until centrifugation, when the blood was spun down, plasma removed and then stored in -20°C until the assay was performed.

To quantify antibody responses to SRBCs, a hemagglutination test was performed on blood plasma. In a 96 well plate, 20 uL of plasma was added to 20 uL of 2% SRBCs diluted in PBS. Eight log2 serial dilutions were performed for each pre-injection plasma sample, and post-injection plasma samples were serially diluted 12 times to ensure we captured individuals with high titers in response to injection. Each plate contained a control row where SRBCs were serially diluted with PBS alone. Plates were then incubated at 37º for 60 minutes and placed on a mirror stand to score for the presence or absence of hemagglutination. The last serial dilution with visibly detectable agglutination was considered the titer for the sample. After initial scoring, wells were vortexed and incubated at room temperature overnight. After 24 hours, plates were rescored. Antibody titer was calculated as the change in titer score from pre- to post-injection, calculated for both 1 hour and 24-hour data separately. Because results were comparable for each timepoint, we only present results for titers at 1 hour.

Data Analysis

Data were analyzed using R Studio version 3.6.2. We used general linear models to ask whether house finch populations (categorical) differed in a suite of responses to immune antigens. All data were modeled assuming a gaussian distribution after looking at model residuals and other diagnostic plots. For some assays (i.e., PHA, SRBC), all individuals received an antigen treatment and served as their own control (post-treatment minus pre-treatment value); thus population alone was the fixed effect of interest in those analyses. For others (i.e., LPS and FSL-1), population in interaction with antigen treatment (control or antigen-injected) was our key variable of interest. Although population was considered a categorical variable for all analyses, results were qualitatively similar when population was treated in an ordinal fashion, from shortest to longest coevolutionary history with MG. Further, because the populations group into two distinct MG exposure histories, short (0-5 years, HI and AZ populations) versus long (20+ years, IA and VA populations), we also ran the analyses grouping populations into these exposure histories.

However, the results were qualitatively similar, and thus we only present results by categorical population here. In addition to the population effects of interest, a variety of other potentially important covariates such as sex were initially included in all models, both alone and, where relevant, in interaction with treatment. Because some birds in our assays had previously been used in an MG infection study, infection history (any prior exposure to MG or no prior exposure) and MG treatment (dose and isolate virulence, if previously exposed) were initially included in starting models to account for any possibility that previous infection could have affected the immune responses measured. Finally, body mass, alone and in interaction with population, was initially included as a covariate in all analyses because the populations differed in average body mass (Figure 1; likelihood ratio $X^2 > 27.6$, d.f.= 3, population: $p < 0.001$; mass values presented from experiment days 0-4). For cloacal temperatures taken in response to injection with LPS and FSL-1, a number of thermocouplers and personnel were used to maximize the quantity of data collected within three minutes of room entry. Thus, in addition to the standard covariates noted above, we also examined potential effects of equipment used for a given bird (thermocoupler ID) and the ID of the individual that took the temperature.

Final models were selected by examining AIC values to determine which model was the best fit. The top five models (i.e., those with the lowest AIC values) then the model with the highest R^2 value was chosen. MG infection history generally did not alter the measured immune responses, and thus this variable was only retained in one final model (see *Results*). Similarly, equipment and personnel used to collect temperature did not alter the measured responses and were not retained in any final model. All p-values were calculated using the car package (Fox & Weisberg, 2018), and the package emmeans was used to conduct all post-hoc tests (Lenth, 2020). Graphs were made using the package ggplot2 (Wickham, 2016).

Results

Whole-Organism Acute Phase Responses

Post-injection mass change. Injection with both LPS and FSL-1 significantly reduced the degree of mass gain relative to controls over six hours post-injection (treatment: $X^2 > 8.9$, df = 1, both $p < 0.01$, Figure 2 and 3). However, for both antigens, the strength of treatment effect did not vary by population as predicted (treatment*population: $X^2 > 2.1$, df = 3, both $p > 0.26$). Pre-injection mass was retained in the best fit models and significantly predicted mass change over six hours post-injection ($X^2 = 10.6$, df = 3, $p = 0.01$), with injected birds that had been previously exposed to MG showing a greater decrease in mass than birds with no previous MG exposure. At 24-hours post-injection, there was no longer a significant effect of either antigen on change in mass (treatment: $X^2 > 2.8$, df = 1, both $p > 0.07$), suggesting that mass effects were relatively short-lived. There was no also population interaction with treatment on change in mass at the 24-hour post-injection measurement (treatment*population: $X^2 > 31.33$, df = 3, both $p > 0.10$).

Cloacal temperature. There were no significant effects of LPS or FSL-1 treatment, either alone or in interaction with population, on the change in cloacal temperatures over six hours post-injection (treatment $X^2 > 2.0$, df = 1, both $p > 0.06$); Figure 4). However, average changes in cloacal temperatures were higher for LPS-injected birds (mean ± s.d.: 0.6 ± 1.5) relative to controls (mean ± s.d.: -0.11 ± 1.6).

Local inflammation in response to PHA

The populations differed significantly in the strength of their swelling response to PHA injection (population: $X^2 > 21.3$ df = 3, $p = 0.01$, Figure 5). The best fit model accounted for individual mass ($X^2 = 0.005$, df =1, $p = 0.67$), the interaction between population and mass ($X^2 = 0.35$, df = 3, $p = 0.01$; Figure 6), and date of injection ($X^2 = 0.7$, df = 2, $p < 0.001$). The average swelling responses were highest for birds from HI and AZ, consistent with our predictions. However, when post-hoc tests were run, there were no significant pair-wise differences between the populations (all $p > 0.13$).

Antibody responses to SRBCs

There was no significant population effect for antibody titer in response to SRBC injection (population: $X^2 > 1.09$, df = 3, p = 0.78, Figure 7).

Discussion

We challenged house finches from four populations spanning distinct MG exposure histories with a suite of immune antigens to detect possible immunological trade-offs that may have arisen from the documented evolution of tolerance and resistance to MG in this species. We used assays that invoked both adaptive and innate immune components to try and broadly examine the possible downstream immunological consequences that may have arisen from the evolution of either disease strategy. Because prior work suggested a potential mechanism of dampened inflammation for evolved tolerance in this system (Adelman et al., 2013), we hypothesized that populations with longer MG exposure histories (i.e., IA and VA populations) would have a reduced inflammatory response to the variety of immune challenges that were conducted during this study. Overall, however, we found little evidence of downstream effects of the evolution of host responses to MG on house finch immune systems more broadly. This suggests that the evolution of host immunological responses to MG in house finches may have occurred in a way that was largely specific to this particular pathogen.

Several of our immune treatments were designed to assay components of the APR, a systemic part of the innate immune response which includes anorexia (causing mass loss) and fever (change in temperature). Given prior work in house finches showing dampened anorexia (i.e., lower mass loss) during MG infection in populations with longer exposure history (Adelman et al., 2013), we hypothesized that birds from populations with evolved MG tolerance would show reduced mass loss and fever in comparison to birds from populations with no evolved tolerance. Further, we expected this pattern to be strongest for FSL-1, given that this antigen stimulates inflammatory responses via the same TLR as MG. However, we did not detect any significant differences across populations in the degree of mass change post-injection

for either antigen (LPS and FSL-1). Although prior work detected dampened mass loss as a potential component of evolved tolerance to MG (Adelman et al., 2013), more recent work with the same populations assayed here did not detect population differences in mass loss in response to MG infection (Bonneaud, Balenger, Hill, & Russell, 2012; Henschen et al., in prep). Thus, if the evolution of MG tolerance was not, in fact, associated with dampened anorexia in house finches, it is unlikely that evolved tolerance to MG would have downstream impacts on anorexia in response to other antigens. However, there are other potential reasons why we may not have detected population differences in mass responses to LPS, even if present. Even though we controlled exposure dose in our study, because LPS is a protein found within all Gram-negative bacteria, it is plausible that the wild-caught house finches used here had previous exposure to this antigen (Benskin, Wilson, Jones, & Hartley, 2009; Morishita, Ley, & Harr, 1999). Because of previous exposure, it is possible that the dose used, though a common dose for passerine studies (Adelman, Córdoba-Córdoba, Spoelstra, Wikelski, & Hau, 2010; Moyers, Kosarski, Adelman, & Hawley, 2015), was not high enough to trigger a sufficiently strong APR to result in detectable population differences. Indeed, there were no longer detectable differences in mass change between control and LPS treatment birds at 24 hours post-injection, whereas other studies of passerines often detect significant mass differences at that timepoint (Owen-Ashley et al., 2006), though those effects can vary with photoperiod in some species (Owen-Ashley, Hasselquist, Råberg, & Wingfield, 2008).

In contrast to LPS, the birds in our study were unlikely to have had prior environmental exposure to FSL-1, which comes from *Mycoplasma salivarium*, a pathogen that does not standardly infect birds (Morishita et al., 1999). To our knowledge, FSL-1 has not been used in passerine studies to date, but here we used a dose previously used in rat models (Hubschle et al., 2006). While higher doses of FSL-1 may have increased the likelihood of detecting population differences in our study, because work has not been done previously in passerines with this antigen, it is also possible that this antigen simply does not trigger a notable APR in house finches. Nonetheless, we did detect a significant treatment effect on change in mass

at 6H post-injection, suggesting that birds in our study did exhibit some degree of anorexia following FSL-1 injection.

Although both antigen treatments resulted in detectable differences in mass relative to controls at 6H post-injection, we did not detect significant differences in cloacal temperature relative to controls at the same timepoint. Thus, the two antigens used did not appear to produce notable fever in our study, and there were no significant differences in the degree of fever detected across populations. Cloacal temperatures are challenging to measure in birds as they can change rapidly with the stress of handling (Cabanac & Aizawa, 2000). While we accounted for this by taking cloacal temperatures within three minutes of room entry, due to these timing constraints, we were only able to robustly measure cloacal temperatures for a subset of birds from each population in response to LPS and FSL-1 injection. Thus, it is possible that the total sample size for cloacal temperatures was not large enough to capture statistically significant treatment effects or population differences in the degree of fever. While higher sample sizes may improve our ability to examine differences in temperature change in response to LPS, for FSL-1 injected birds, cloacal temperatures generally decreased or stayed the same post-injection. Because use of FSL-1 has not yet been documented in passerines or chickens, it is possible that while it can cause fever in mammalian models (Greis, Murgott, Gerstberger, Hübschle, & Roth, 2009), the dose or the immune response it produces in birds does not result in fever.

In addition to measuring components of the APR, we also assayed a metric of localized inflammation (wing web swelling) in response to PHA injection. We detected small but statistically significant population differences in swelling responses relative to body mass. Heavier individuals generally showed stronger swelling responses, a pattern also detected in prior studies of peacocks (Møller & Petrie, 2002), but the strength of the relationship between body mass and swelling responses varied across populations. When these complex effects of mass were accounted for, populations that had shorter exposure histories to MG (HI and AZ), and thus no evolved MG tolerance, had a greater swelling response to PHA than birds with a longer exposure history to MG, consistent with our predictions. However, because the pattern

of reduced swelling response in populations with evolved tolerance is correlational, and was only seen when population and individual differences in body mass were taken into account, we are unable to definitively tease apart whether the detected population differences result from confounding variables or are a result of downstream effects of evolved tolerance to MG. If MG exposure history does play a role in the detected differences in swelling response, it is possible that these differences arose because the inflammatory mechanisms that are used to respond to PHA share similarities with the mechanisms that underlie conjunctivitis in response to MG. Injection with PHA causes a very generalized immune response; after injection, leukocytes are recruited to the area, which causes the localized swelling (Bílková et al., 2015; Vinkler et al., 2010). It is thought that the tolerance mechanisms that evolved to mediate MG operate through a reduction in conjunctival inflammation (Adelman et al., 2013), which can also include leukocyte recruitment (Hawley, Grodio, Frasca Jr, Kirkpatrick, & Ley, 2011). It is thus possible that the localized immune responses following PHA injection are more similar to the conjunctival responses induced by MG infection than are the whole-organism responses stimulated by LPS and FSL-1. If so, this could help explain why this is the only immune assay where significant population differences were seen. However, given the correlational nature of the patterns detected here and the potential confounding influence of mass, future work should probe this relationship further to confirm causality and examine to what extent similar immunological mechanisms underlie PHA and MG responses in house finches.

In addition to examining several components of the innate immune response, we also examined whether there were population-level differences in antibody titers in response to SRBC injection using a hemagglutination assay. Because prior work in chickens demonstrated that artificial selection for SRBC antibody responses led to a concomitant increase in resistance to MG (Gross et al., 1980), we predicted that birds with longer exposure histories to MG and thus evolved MG resistance would have increased antibody production in response to SRBC injection. However, we did not find any evidence that the populations differed in their degree of antibody response to SRBCs. This could be for several reasons.

First, while evidence consistent with the evolution of MG resistance has been demonstrated in this system (Bonneaud et al., 2011; Bonneaud et al., 2018), other studies have not detected any evidence of resistance (Adelman et al., 2013). Thus, selection on tolerance in this system may have been stronger than selection on resistance, the latter of which would be more likely to involve antibody responses (Bonneaud et al., 2006). In addition, antibody production is highly variable and plastic, responding to stimuli through many mechanisms (Abbas, Lichtman, & Pillai, 2014). Finally, while there is some evidence that MG-specific antibody responses can be protective in this system (Fleming-Davies et al. 2018), it remains unknown whether antibody responses to MG are associated with the detected evolution of resistance in this system (Bonneaud et al., 2011). Further, because of the many mechanisms that are used to produce antibodies, this makes it even more unlikely that evolution in response to one pathogen could drastically alter antibody production to another (Baumgarth, Tung, & Herzenberg, 2005), except perhaps in cases where strong artificial selection is placed on immune responses, as was done in the chicken SRBC-MG study (Gross et al., 1980).

The overall purpose of this study was to examine the possible immunological trade-offs that may have arisen as tolerance or resistance to MG evolved in house finches. Although we used a suite of assays that involved diverse components of the immune system, the only potential trade-off that was detected in this study was a reduced swelling response after PHA injection in populations with evolved tolerance to MG. Since previous work (Henschen et al., in prep) demonstrated that eastern house finch populations (VA and IA) have evolved both tolerance and resistance to MG, it is interesting that our work using methods commonly employed in ecological immunology studies did not capture more notable differences in immunological responses between the populations. Overall, this disconnect suggests that immune assays, while useful in many respects, may not adequately capture meaningful immunological variation in response to ecologically-relevant pathogens in wild populations (Calisi & Bentley, 2009). Additionally, there was extensive heterogeneity in immune responses within populations, which could have prevented us from seeing trade-offs that might be present. All of the house finches used in this experiment were

caught from the wild and, because of this, have diverse immunological and developmental histories (food availability, and stress) (Hoi-Leitner, Romero-Pujante, Hoi, & Pavlova, 2001; Suorsa et al., 2004). Given this high variability, we may have needed larger sample sizes to robustly test the predictions of interest.

Overall, we found only limited support for broader immunological effects of the evolution of host responses to MG in house finches. Although work across a suite of systems using artificial selection suggests that the evolution of resistance to a parasite or antigen is often associated with effects on other responses (Fellowes, Kraaijeveld, & Godfray, 1998; Fellowes et al., 1999), our results were largely unable to detect strong immunological differences associated with the evolution of tolerance and resistance. This suggests that the evolution of host immunological responses to MG in house finches may have occurred in a way that was largely specific to this particular pathogen. On the other hand, it is also possible that the populations under study do differ more markedly in their broader immunological responses, but the assays used in this experiment did not stimulate the components of the immune response associated with population differences. For example, previous work across house finch populations (Adelman et al., 2013) and pathogen strains (Vinkler et al., 2018) examined cytokine expression in response to MG, which may be more likely to show population differences associated with evolved tolerance in this system. Additionally, it would be interesting to perform experimental infections with pathogens other than MG, as host responses to live infections may be more likely to capture meaningful immunological differences than can the injection of inert antigens.

Further work on the immunological mechanisms associated with both tolerance and resistance to MG in this system, which are currently largely unknown, will help direct future studies on potential downstream immunological effects of the evolution of host responses to MG in house finches. These studies will be critical for elucidating whether the evolution of host responses in natural systems, as has occurred in house finches, results in detectable immunological effects on host responses to other antigens. Our study suggests that the effects may be minimal in this system, but studies on a suite of natural systems are needed before robust conclusions can be drawn. Given the critical implications that evolutionary

immunological trade-offs can have both ecological (e.g., coinfection) and evolutionary (e.g., coevolution) interactions between hosts and their pathogens, future studies on natural systems with evolved tolerance or resistance to emerging pathogens are sorely needed.

Figures

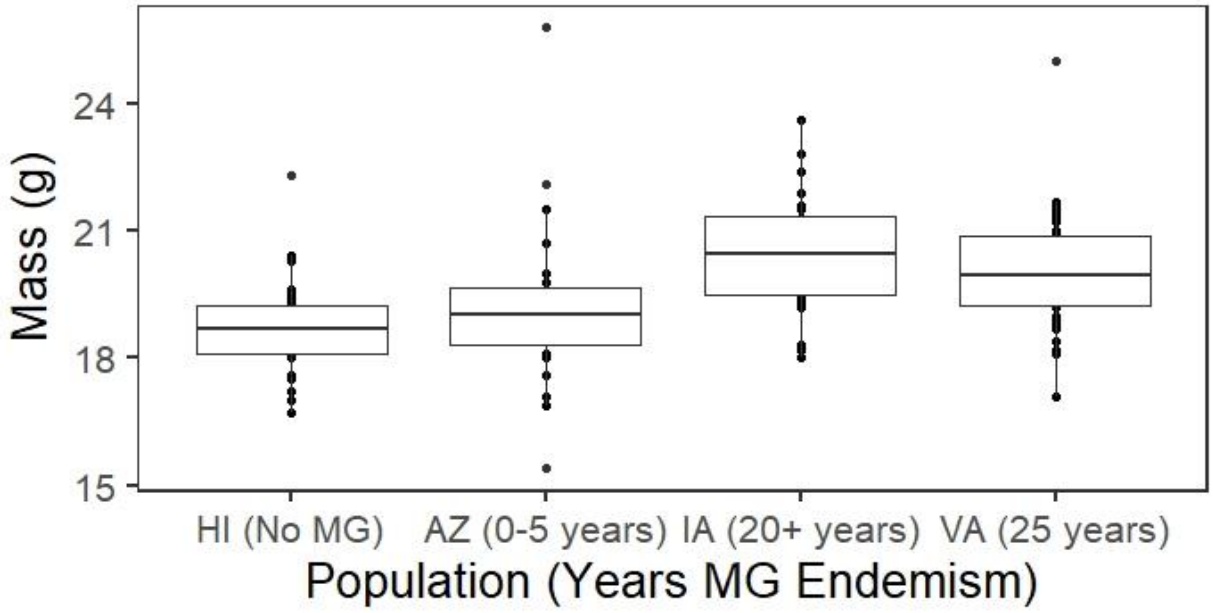

Figure 1. House finch populations that vary in their history of exposure to *Mycoplasma gallisepticum* (MG) differed significantly in baseline body mass before any immune challenges were conducted. Birds from HI (18.7 g ± s.d 1.1) and AZ (19.1 g ± s.d 1.5) were smaller on average than those from VA (20.1 g ± s.d 1.4) and IA (20.4 g ± s.d 1.4). In pairwise post-hoc tests, HI populations were significantly different from IA and VA ($p < 0.05$), and AZ was significantly different from IA ($p= 0.0048$) and almost significantly different from VA ($p = 0.057$). The data presented were collected just prior to PHA injection, on experimental days 0–4, but similar patterns were present throughout the study.

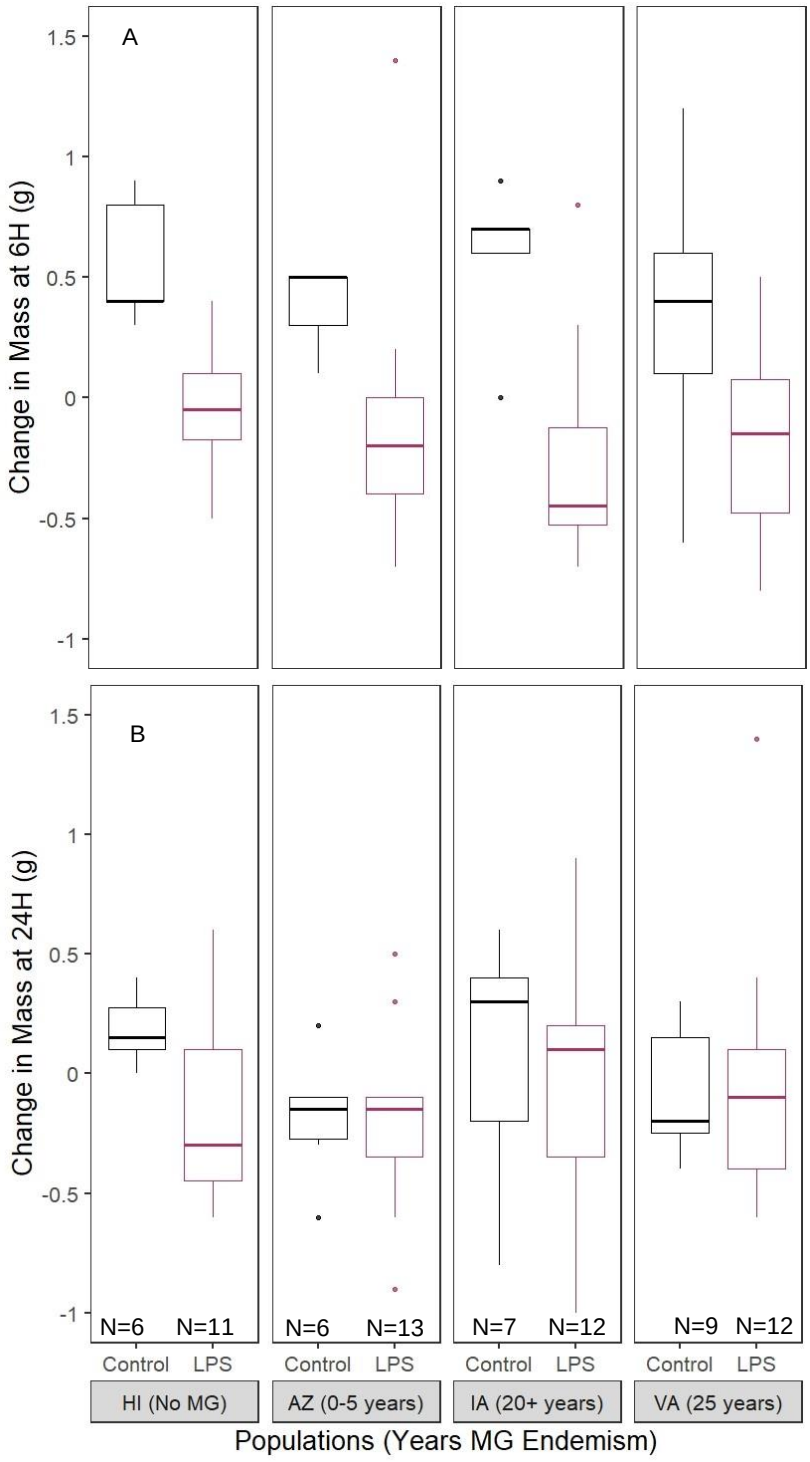

Figure 2. Change in mass (means and quartiles) following LPS injection (A: 6 hours post-injection, B: 24-hours post-injection) or control treatment for house finches from one of four populations that vary in their history of exposure to *Mycoplasma gallisepticum* (MG). While LPS treatment significantly predicted mass loss at 6H post-injection, the effects of treatment did not differ across populations at either time point.

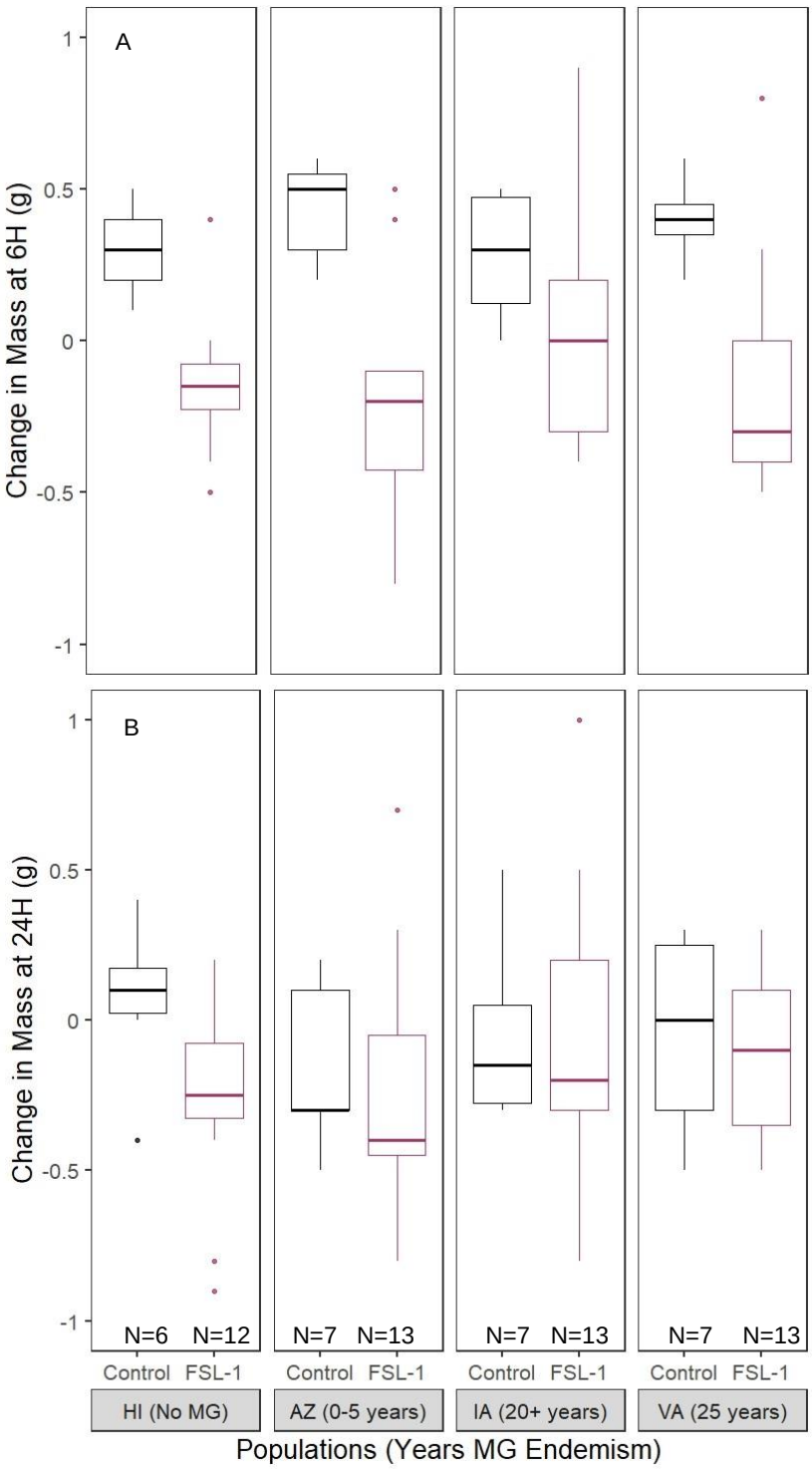

Figure 3. Change in mass (means and quartiles) following FSL-1 injection (A: 6 hours (H) post-injection, B: 24H post-injection) or control treatment for house finches from one of four populations that vary in their history of exposure to *Mycoplasma gallisepticum* (MG).While treatment with FSL-1 was a significant predictor of mass 6H post-injection, the effects of treatment did not differ across populations.

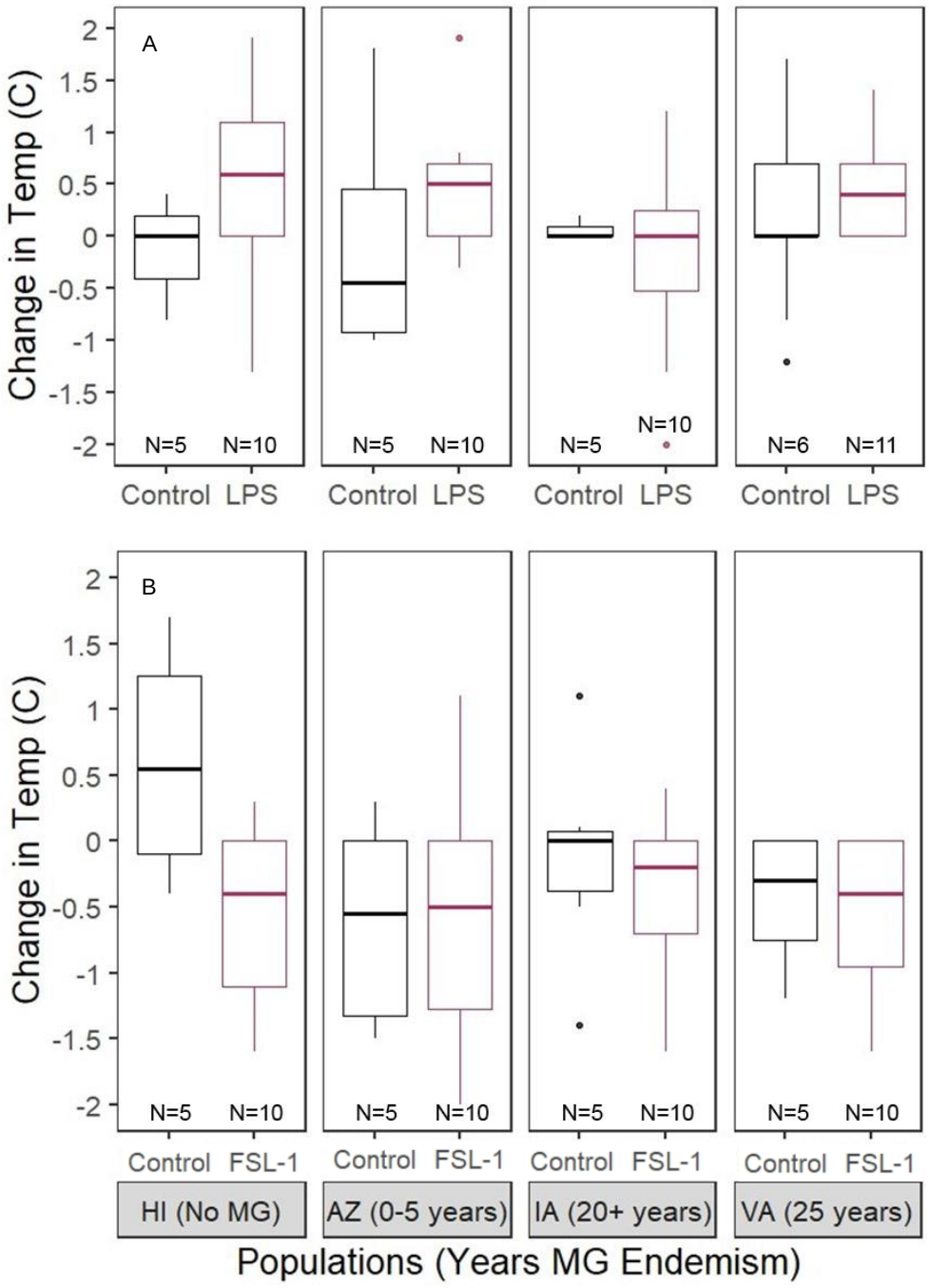

Figure 4. Changes in cloacal temperature (means and quartiles) six hours post-injection with one of two antigens (A: FSL-1, B: LPS) or control treatment in house finches from one of four populations that vary in their history of exposure to *Mycoplasma gallisepticum* (MG). There were no significant treatment effects or population differences detected.

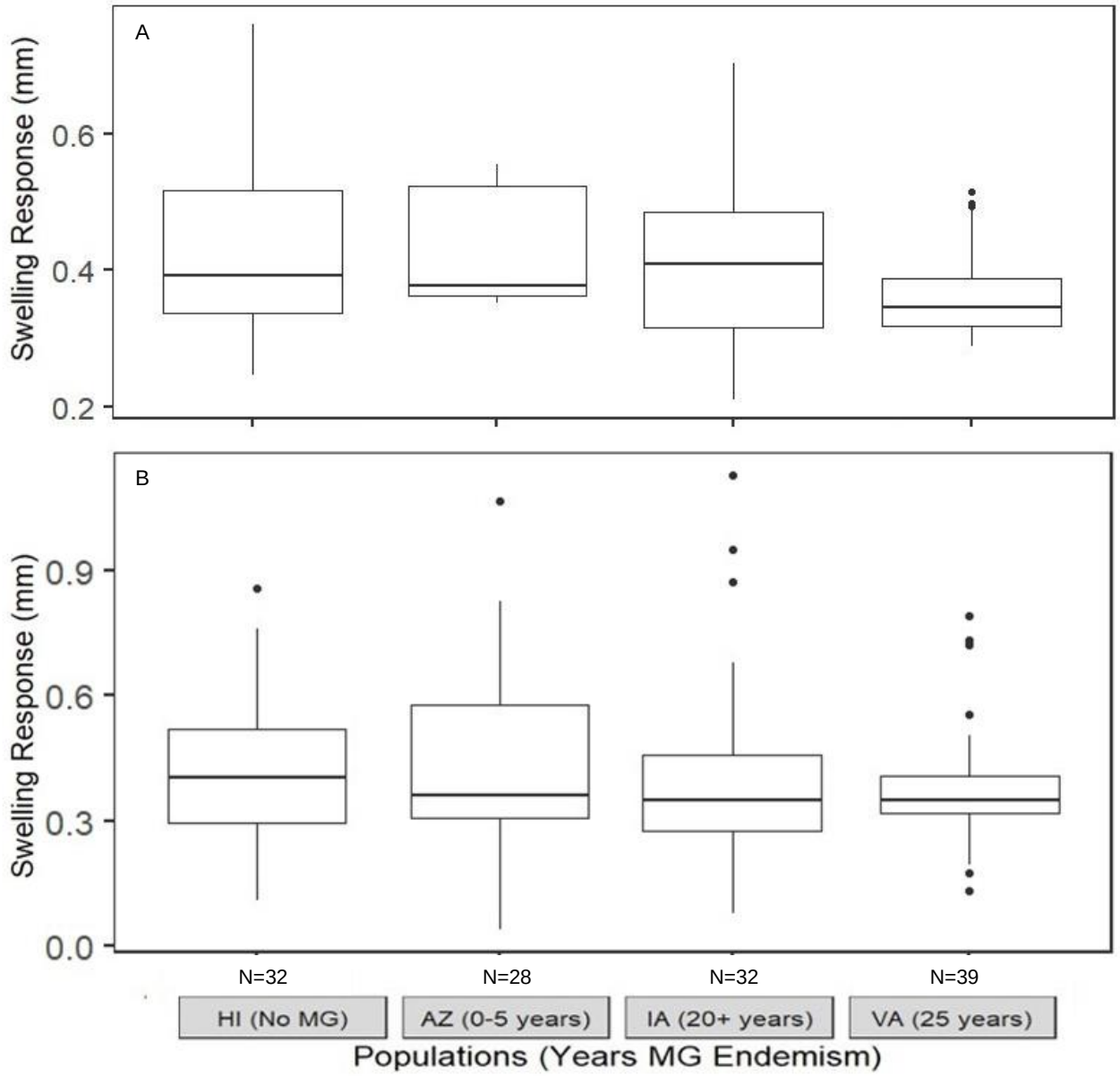

Figure 5. Populations that vary in their history of exposure to *Mycoplasma gallisepticum* (MG) significantly differed in the swelling response (means and quartiles) in response to PHA injection. Swelling responses are graphed both as (A) predicted values from the best fit model that accounted for individual mass, sex, and a significant interaction between population and mass, and (B) as raw data.

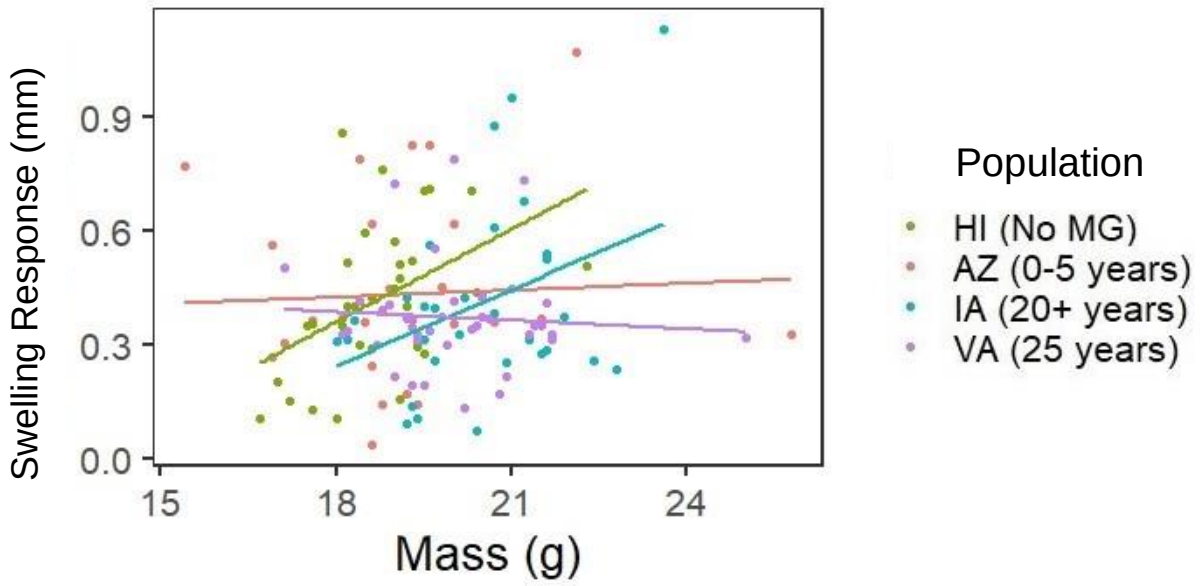

Figure 6. The relationship between house finch mass (g) and swelling response (mm) across four populations that vary in their history of exposure to *Mycoplasma gallisepticum* (MG), with best fit lines for the relationship between mass and swelling response within each population. Raw data are graphed.

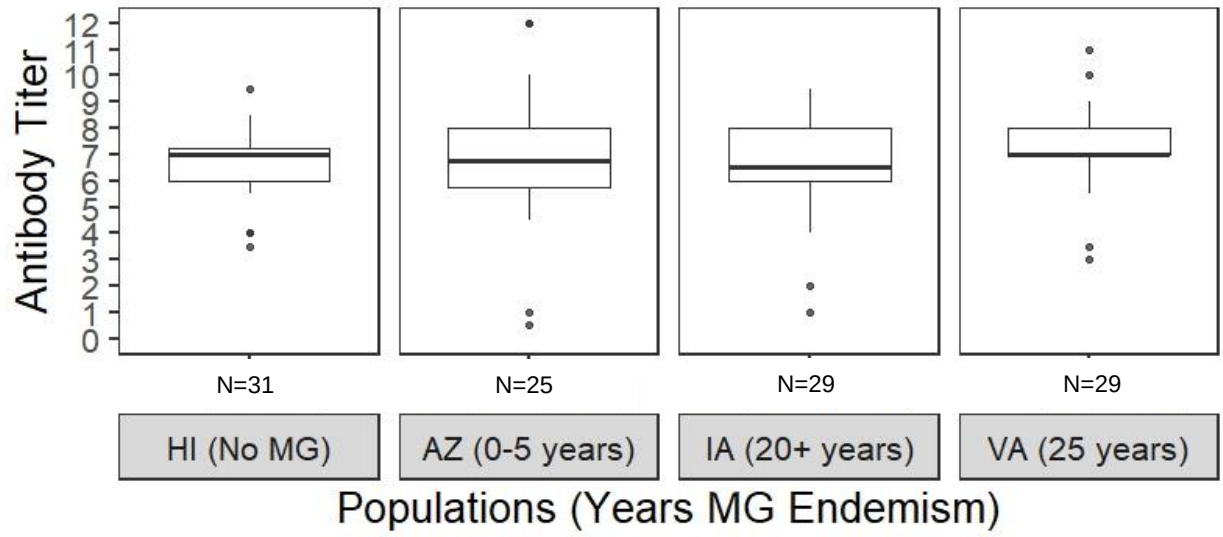

Figure 7. Antibody titers (means and quartiles) in response to SRBC injection did not differ across four populations of house finches that vary in their history of exposure to *Mycoplasma gallisepticum* (MG).

Chapter 3 - Conclusion

The purpose of this study was to examine what possible immunological trade-offs might have arisen as house finches (*Haemorhous mexicanus)* evolved tolerance to *Mycoplasma gallisepticum* (MG). Originally found in domestic poultry, MG spilled over into eastern North American house finch populations and then spread throughout the continental United States. As MG spread, different geographic populations of house finches encountered MG initially at different periods of time. These differences allow host evolution in response to MG to be examined using a space-for-time approach, and these studies revealed that populations with a longer exposure history with MG have evolved disease tolerance and/or resistance to the pathogen (Adelman et al., 2013; Bonneaud et al., 2011; Bonneaud et al., 2019). Four populations with varying exposure history to MG, and thus varying evolved tolerance and resistance, were used for this study, ranging from populations that had been exposed to MG for 25 years (Virginia) to populations that have not yet had any MG exposure (Hawaii). Using a variety of immune assays stimulating both the adaptive and innate immune system, birds from these populations were immunologically challenged so that population immunological differences could parse out where and whether trade-offs may have arisen as populations evolved tolerance and resistance to MG.

The evolution of many traits such as resistance to a parasite or pathogen can result in downstream evolutionary effects (often negative) on other traits important for fitness. With the documented rapid evolution of the house finch immune system to tolerate/resist MG, there are likely broader components of the immune system that are also affected. Part of the motivation behind our work was to tease apart the evolutionarily costs associated with the evolution of host tolerance, including how those costs differ from the costs of resistance. Because the mechanisms of evolved resistance and tolerance are likely inherently different, the evolutionary trade-offs that result from tolerance may be very different than those that result from evolved resistance. As noted in Chapter 1, tolerance may be more likely to evolve through more general, innate immune pathways associated with inflammation or tissue repair. This could have several possible effects on downstream evolutionary trade-offs. First, evolving tolerance through more general

pathways may be more likely to result in consistent evolutionary effects on responses to other parasites and pathogens, potentially by increasing "tolerance" to many types of antigens. On the other hand, general pathways such as the innate immune response may inherently be highly conserved, minimizing the likelihood of evolutionary responses to one pathogen impacting responses to another. Resistance, in contrast, often evolves through specific mechanisms that can increase defense against or killing of a specific pathogen, potentially at the expense of another. Overall, it is highly plausible that the downstream evolutionary effects of tolerance and resistance differ.

Interestingly, work done on the house finch system suggests that both tolerance and resistance have evolved in populations with longer exposure histories to MG (Adelman et al., 2013; Bonneaud et al., 2011; Bonneaud et al., 2019). To account for this, we conducted immune challenges that looked at both the innate immune system (which would likely be more affected by the evolution of tolerance) as well as the adaptive immune system (which would likely be more affected by the evolution of resistance). Because prior work showed that populations that were exposed to MG for longer had a decrease in early inflammatory cytokine expression when infected with MG (Adelman et al., 2013), we hypothesized that populations with evolved tolerance to MG would have a dampened innate inflammatory response. Of the four assays used, the only immune challenge that potentially supported this hypothesis was localized swelling in response to PHA injection, with decreased swelling detected in birds with longer exposure to MG when individual and population differences in mass were accounted for. Since the PHA challenge was the only assay that provided even limited support for our hypothesis, immune responses to PHA and MG may rely on more similar immunological mechanisms than do some of the other immune assays examined. PHA injection causes a localized swelling response through inflammation and recruitment of leukocytes to the injection area (Bílková et al., 2015; Vinkler et al., 2010). Previous work suggests that inflammation reduction is the tolerance mechanism that has evolved in response to MG (Adelman et al., 2013), which may show similarities to PHA in terms of cell recruitment and other localized responses.

However, because of the confounding effects of mass differences within and between populations, the detected differences in PHA responses across populations should be interpreted with caution.

Overall, we did not see strong differences in immunological responses associated with evolved tolerance or resistance in house finches. Components of the innate immune system (such as mass change and fever) that may be less strongly associated with tolerance to MG did not appear to evolve alongside the evolution of host responses to MG. To examine a possible downstream effect of resistance evolution, we examined antibody production in response to sheep red blood cells, but found no population-level differences observed for this assay. However, since no population differences have been seen in the strength of antibody responses to MG (Henschen et al., in prep), it is possible that this is not a pathway that resistance has evolved through in this system, and thus downstream effects on antibody responses to other antigens would not be expected. Previous work addressing evolutionary trade-offs has detected these trade-offs outside of the specific pathway that has evolved (Hague et al., 2018), but in the case of MG tolerance, our work did not find this to be the case. It appears that with MG tolerance, any trade-offs that are present are likely most strongly associated with mechanisms specifically used for tolerating MG. However, the exact mechanisms underlying tolerance to MG are still unknown, and thus future work should continue to examine the inflammatory pathways that are likely affected through the evolution of MG tolerance. A better understanding of the pathways that are affected would allow for a more precise examination of where evolutionary trade-offs might arise.

To better tease apart what immunological trade-offs might be present in house finches with evolved tolerance and resistance, future work should examine more complex immune components such as cytokine expression or host responses to other experimental infections, which require the integration of innate and adaptive immune components. Previous work has found population differences in cytokine responses (Adelman et al., 2013) and further exploring these differences would help provide a more conclusive idea about what pathways are being modified as tolerance evolves. Cytokines are important molecules that help integrate the immune response and modification of cytokine expression could have

large immunological effects if its modification is non-specific. However, it is also possible that cytokine differences seen in response to MG have evolved highly specifically and would not be seen in response to other pathogens. Additionally, cellular profiling would inform to extent to which localized cell recruitment contributes to inflammation in response to MG, and the degree to which these responses have been dampened as tolerance evolved. Currently, immunological tools such as cellular profiling are not available for passerine populations. However as immunological tools for non-model avian systems become more available (Lemus, Vergara, & Fargallo, 2010), a more thorough examination could be done to better tease apart which components of the immune system are responding to the evolution of tolerance. This might better capture the mechanisms that are responding to the evolution of tolerance or resistance and help provide a clearer picture about what trade-offs might be occurring in this system. Lastly, it is entirely possible that the evolution of tolerance to MG is incredibly specific and there might not necessarily be detectable downstream costs or benefits in natural populations of house finches. If the immune system evolved in a very specific way, then immunological effects might not arise when responding to other pathogens, which would be highly advantageous for wild populations. Emerging diseases place strong selection pressures on their hosts, and if hosts are able to evolve strategies to combat these pathogens with minimal downstream consequences, this would allow hosts to reduce the fitness effects of one emerging disease without putting themselves at higher risk for another.

References

Abbas, A. K., Lichtman, A. H., & Pillai, S. (2014). *Cellular and molecular immunology E-book*: Elsevier Health Sciences.

Adelman, J. S., Córdoba-Córdoba, S., Spoelstra, K., Wikelski, M., & Hau, M. (2010). Radiotelemetry reveals variation in fever and sickness behaviours with latitude in a free-living passerine. *Functional Ecology, 24*(4), 813-823.

Adelman, J. S., Kirkpatrick, L., Grodio, J. L., & Hawley, D. M. (2013). House finch populations differ in early inflammatory signaling and pathogen tolerance at the peak of Mycoplasma gallisepticum infection. *The American Naturalist, 181*(5), 674-689.

Ahmad, R., Shihab, P. K., Jasem, S., & Behbehani, K. (2014). FSL-1 induces MMP-9 production through TLR-2 and NF-κB/AP-1 signaling pathways in monocytic THP-1 cells. *Cellular Physiology and Biochemistry, 34*(3), 929-942.

Alves, J. M., Carneiro, M., Cheng, J. Y., de Matos, A. L., Rahman, M. M., Loog, L., . . . Manica, A. (2019). Parallel adaptation of rabbit populations to myxoma virus. *Science, 363*(6433), 1319-1326.

Baumann, H., & Gauldie, J. (1994). The acute phase response. *Immunology today, 15*(2), 74-80.

Baumgarth, N., Tung, J. W., & Herzenberg, L. A. (2005). *Inherent specificities in natural antibodies: a key to immune defense against pathogen invasion.* Paper presented at the Springer seminars in immunopathology.

Benskin, C. M. H., Wilson, K., Jones, K., & Hartley, I. R. (2009). Bacterial pathogens in wild birds: a review of the frequency and effects of infection. *Biological Reviews, 84*(3), 349-373.

Bílková, B., Vinklerová, J., & Vinkler, M. (2015). The relationship between health and cell-mediated immunity measured in ecology: Phytohaemagglutinin skin-swelling test mirrors blood cellular composition. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology, 323*(10), 767-777.

Bonneaud, C., Balenger, S. L., Hill, G. E., & Russell, A. F. (2012). Experimental evidence for distinct costs of pathogenesis and immunity against a natural pathogen in a wild bird. *Molecular ecology, 21*(19), 4787-4796.

Bonneaud, C., Balenger, S. L., Russell, A. F., Zhang, J., Hill, G. E., & Edwards, S. V. (2011). Rapid evolution of disease resistance is accompanied by functional changes in gene expression in a wild bird. *Proceedings of the National Academy of Sciences, 108*(19), 7866-7871.

Bonneaud, C., Balenger, S. L., Zhang, J., Edwards, S. V., & Hill, G. E. (2012). Innate immunity and the evolution of resistance to an emerging infectious disease in a wild bird. *Molecular ecology, 21*(11), 2628-2639.

Bonneaud, C., Giraudeau, M., Tardy, L., Staley, M., Hill, G. E., & McGraw, K. J. (2018). Rapid antagonistic coevolution in an emerging pathogen and its vertebrate host. *Current Biology, 28*(18), 2978-2983. e2975.

Bonneaud, C., Pérez-Tris, J., Federici, P., Chastel, O., & Sorci, G. (2006). Major histocompatibility alleles associated with local resistance to malaria in a passerine. *Evolution, 60*(2), 383-389.

Bonneaud, C., Tardy, L., Giraudeau, M., Hill, G. E., McGraw, K. J., & Wilson, A. J. (2019). Evolution of both host resistance and tolerance to an emerging bacterial pathogen. *Evolution Letters*.

Cabanac, M., & Aizawa, S. (2000). Fever and tachycardia in a bird (Gallus domesticus) after simple handling. *Physiology & behavior, 69*(4-5), 541-545.

Calisi, R. M., & Bentley, G. E. (2009). Lab and field experiments: are they the same animal? *Hormones and behavior, 56*(1), 1-10.

Clutton-Brock, T. H., Guinness, F. E., & Albon, S. D. (1982). *Red deer: behavior and ecology of two sexes*: University of Chicago press.

Cotter, S. C., Kruuk, L. E. B., & Wilson, K. (2004). Costs of resistance: genetic correlations and potential trade-offs in an insect immune system. *Journal of evolutionary biology, 17*(2), 421-429.

Dhondt, A. A., Altizer, S., Cooch, E. G., Davis, A. K., Dobson, A., Driscoll, M. J., . . . Hosseini, P. R. (2005). Dynamics of a novel pathogen in an avian host: mycoplasmal conjunctivitis in house finches. *Acta tropica, 94*(1), 77-93.

Faustino, C. R., Jennelle, C. S., Connolly, V., Davis, A. K., Swarthout, E. C., Dhondt, A. A., & Cooch, E. G. (2004). Mycoplasma gallisepticum infection dynamics in a house finch population: seasonal variation in survival, encounter and transmission rate. *Journal of Animal Ecology, 73*(4), 651-669.

Fellowes, M., Kraaijeveld, A., & Godfray, H. (1998). Trade–off associated with selection for increased ability to resist parasitoid attack in Drosophila melanogaster. *Proceedings of the Royal Society of London. Series B: Biological Sciences, 265*(1405), 1553-1558.

Fellowes, M., Kraaijeveld, A., & Godfray, H. (1999). Cross-resistance following artificial selection for increased defense against parasitoids in Drosophila melanogaster. *Evolution, 53*(3), 966-972.

Fox, J., & Weisberg, S. (2018). *An R companion to applied regression*: Sage publications.

Greis, A., Murgott, J., Gerstberger, R., Hübschle, T., & Roth, J. (2009). Effects of repeated injections of fibroblast-stimulating lipopeptide-1 on fever, formation of cytokines, and on the responsiveness to endotoxin in guinea-pigs. *Acta physiologica, 197*(1), 35-45.

Gross, W., Siegel, P., Hall, R., Domermuth, C., & DuBoise, R. (1980). Production and persistence of antibodies in chickens to sheep erythrocytes. 2. Resistance to infectious diseases. *Poultry science, 59*(2), 205-210.

Gustafsson, L., Nordling, D., Andersson, M., Sheldon, B., & Qvarnström, A. (1994). Infectious diseases, reproductive effort and the cost of reproduction in birds. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences, 346*(1317), 323-331.

Hague, M. T., Toledo, G., Geffeney, S. L., Hanifin, C. T., Brodie Jr, E. D., & Brodie III, E. D. (2018). Large-effect mutations generate trade-off between predatory and locomotor ability during arms race coevolution with deadly prey. *Evolution Letters, 2*(4), 406-416.

Hawley, D. M., Grodio, J., Frasca Jr, S., Kirkpatrick, L., & Ley, D. H. (2011). Experimental infection of domestic canaries (Serinus canaria domestica) with Mycoplasma gallisepticum: a new model system for a wildlife disease. *Avian Pathology, 40*(3), 321-327.

Henschen, A. E., Adelman, J. S., Hawley, D. M., Dalloul, R. A., Rowley, A. A., & Langager, M. M. (in prep). Evolved Disease Tolerance in House Finches.

Hochachka, W. M., & Dhondt, A. A. (2000). Density-dependent decline of host abundance resulting from a new infectious disease. *Proceedings of the National Academy of Sciences, 97*(10), 5303-5306.

Hoi-Leitner, M., Romero-Pujante, M., Hoi, H., & Pavlova, A. (2001). Food availability and immune capacity in serin (Serinus serinus) nestlings. *Behavioral Ecology and Sociobiology, 49*(5), 333-339.

Hubschle, T., Mutze, J., Muhlradt, P. F., Korte, S., Gerstberger, R., & Roth, J. (2006). Pyrexia, anorexia, adipsia, and depressed motor activity in rats during systemic inflammation induced by the Toll-like receptors-2 and-6 agonists MALP-2 and FSL-1. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 290*(1), R180-R187.

Kushner, I. (1982). The phenomenon of the acute phase response. *Annals of the New York Academy of Sciences, 389*(1), 39-48.

Langwig, K. E., Hoyt, J. R., Parise, K. L., Frick, W. F., Foster, J. T., & Kilpatrick, A. M. (2017). Resistance in persisting bat populations after white-nose syndrome invasion. *Philosophical Transactions of the Royal Society B: Biological Sciences, 372*(1712), 20160044.

Lemus, J. A., Vergara, P., & Fargallo, J. A. (2010). Response of circulating T-lymphocytes to a coccidian infection: insights from a parasitization-vaccination experiment. *Functional Ecology, 24*(3), 638-645.

Lenth, R. (2020). emmeans: Estimated Marginal Means, aka Least-Squares Means. Retrieved from https://CRAN.R-project.org/package=emmeans

Ley, D. H., Berkhoff, J. E., & McLaren, J. M. (1996). Mycoplasma gallisepticum isolated from house finches (Carpodacus mexicanus) with conjunctivitis. *Avian diseases, 40*(2), 480-483.

Majumder, S., Zappulla, F., & Silbart, L. K. (2014). Mycoplasma gallisepticum lipid associated membrane proteins up-regulate inflammatory genes in chicken tracheal epithelial cells via TLR-2 ligation through an NF-κB dependent pathway. *PLoS One*, *9*(11), e112796.

Medzhitov, R., Schneider, D. S., & Soares, M. P. (2012). Disease tolerance as a defense strategy. *Science, 335*(6071), 936-941.

Møller, A. P., & Petrie, M. (2002). Condition dependence, multiple sexual signals, and immunocompetence in peacocks. *Behavioral Ecology*, *13*(2), 248-253.

Morishita, T. Y., Ley, E. C., & Harr, B. S. (1999). Survey of pathogens and blood parasites in free-living passerines. *Avian diseases*, 549-552.

Moyers, S. C., Kosarski, K. B., Adelman, J. S., & Hawley, D. M. (2015). Interactions between social behaviour and the acute phase immune response in house finches. *Behaviour, 152*(15), 2039-2058.

Nestor, K. E., Noble, D., Zhu, J., & Moritsu, Y. (1996). Direct and correlated responses to long-term selection for increased body weight and egg production in turkeys. *Poultry science, 75*(10), 1180-1191.

Owen-Ashley, N. T., Hasselquist, D., Råberg, L., & Wingfield, J. C. (2008). Latitudinal variation of immune defense and sickness behavior in the white-crowned sparrow (Zonotrichia leucophrys). *Brain, behavior, and immunity*, *22*(4), 614-625.

Owen-Ashley, N. T., Turner, M., Hahn, T. P., & Wingfield, J. C. (2006). Hormonal, behavioral, and thermoregulatory responses to bacterial lipopolysaccharide in captive and free-living white-crowned sparrows (Zonotrichia leucophrys gambelii). *Hormones and behavior*, *49*(1), 15-29.

Råberg, L., Sim, D., & Read, A. F. (2007). Disentangling genetic variation for resistance and tolerance to infectious diseases in animals. *Science*, *318*(5851), 812-814.

Saino, N., Calza, S., & Møller, A. P. (1998). Effects of a dipteran ectoparasite on immune response and growth trade-offs in barn swallow, Hirundo rustica, nestlings. *Oikos*, 217-228.

Savage, A. E., & Zamudio, K. R. (2016). Adaptive tolerance to a pathogenic fungus drives major histocompatibility complex evolution in natural amphibian populations. *Proceedings of the Royal Society B: Biological Sciences*, *283*(1827), 20153115.

Schwenke, R. A., Lazzaro, B. P., & Wolfner, M. F. (2016). Reproduction–immunity trade-offs in insects. *Annual Review of Entomology*, *61*, 239-256.

Staley, M., Bonneaud, C., McGraw, K. J., Vleck, C. M., & Hill, G. E. (2017). Detection of Mycoplasma gallisepticum in house finches (Haemorhous mexicanus) from Arizona. *Avian diseases, 62*(1), 14-17.

Stearns, S. C. (1992). *The evolution of life histories*.

Suorsa, P., Helle, H., Koivunen, V., Huhta, E., Nikula, A., & Hakkarainen, H. (2004). Effects of forest patch size on physiological stress and immunocompetence in an area-sensitive passerine, the Eurasian treecreeper (Certhia familiaris): an experiment. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *271*(1537), 435-440.

Vinkler, M., Bainová, H., & Albrecht, T. (2010). Functional analysis of the skin-swelling response to phytohaemagglutinin. *Functional Ecology*, *24*(5), 1081-1086.

Vinkler, M., Leon, A. E., Kirkpatrick, L., Dalloul, R. A., & Hawley, D. M. (2018). Differing house Finch cytokine expression responses to Original and evolved isolates of Mycoplasma gallisepticum. *Frontiers in immunology*, *9*, 13.

Wickham, H. (2016). *ggplot2: elegant graphics for data analysis*: Springer.

Ye, Y. H., Chenoweth, S. F., & McGraw, E. A. (2009). Effective but costly, evolved mechanisms of defense against a virulent opportunistic pathogen in Drosophila melanogaster. *PLoS Pathogens, 5*(4), e1000385-e1000385.