
# The evolution and protective benefits of immune responses in North American house finches (*Haemorhous mexicanus*) to *Mycoplasma gallisepticum*

Submitted by Daisy Gates to the University of Exeter as a thesis for the degree of Masters by Research in Biological Sciences, May 2018.

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# Thesis Abstract

Pathogen spillover to novel hosts represents a huge selective event, and can result in rapid evolutionary changes in both the pathogen and the host. However, it is seldom possible to identify which specific host characteristics are under selection following emergence, nor understand the evolutionary repercussions of these changes. In the early 1990's, *Mycoplasma gallisepticum* emerged in North American house finches (*Haemorhous mexicanus*) following a jump from poultry. Previous studies monitoring disease spread and response to infection provided details of temporal and geographic patterns of host history of exposure, including evidence of the evolution of resistance in populations with a long history of exposure. In this thesis, I explore the specific host responses that were subject to selection following disease emergence in coevolved, relative to unexposed populations and evaluate how this led to the qualitative disease dynamics previously observed. In chapter one, I show that susceptibility to infection must be equal in both susceptible and resistant host genotypes in order for selection to occur, with mortality avoidance most likely driving the mode and tempo of rapid selection on resistance and virulence evolution observed in this system. In the second chapter, I find limited evidence for a protective benefit of a commonly measured immune component: systemic antibody, and show other important immune processes that are involved in host resistance, including an avoidance of immune manipulation. Findings presented here demonstrate the importance of the specific mechanism of host responses in determining evolutionary trajectories.

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# Chapter 1: General Introduction

Infectious diseases are important selective agents in a range of species, including humans (Karlsson *et al.*, 2014), and have had devastating effects on biodiversity due to their contribution to recent species declines (Lips *et al.*, 2006; Pounds *et al.*, 2006). Interpreting the protective benefits of host responses to infection is complex, particularly in scenarios where pathogens are able to manipulate host responses and cause damage through immunopathology. Furthermore, external or unrelated ecological factors including, but not limited to: host condition (Cornet *et al.*, 2014), resource availability (Pedersen & Greives, 2008) and climate (Atkinson *et al.*, 2014) can all influence the strength and type of response elicited, due to physiological and energetic trade offs with other biological processes (Hawley *et al.*, 2012; Cheatsazan *et al.*, 2013).

An additional layer of complexity facing ecologists and evolutionary biologists is that hosts and pathogens continually adapt to one another, on both spatial and temporal scales (Lively & Dybdahl, 2000; Decaestecker *et al.*, 2007) and therefore the immune response is a dynamic and responsive process. As all organisms including humans, live in a constantly varying environment, it is therefore important that we aim to understand as much as possible about the ecological interactions of hosts and pathogens in the context of natural populations (Pedersen & Babayan, 2011).

There is growing appreciation that understanding host defenses at the immediate time of infection is crucial for understanding the evolutionary trajectories of host resistance and pathogen evolution (Mackinnon & Read, 2004). This knowledge is useful not only in developing treatments (Gandon *et al.*, 2001), but also for targeting strategies for preventative measures (Griffin & Catlla, 2013). Specifically, the nature of host responses can have a direct impacts on pathogen evolution, influencing strategies for both transmission and the level of damage they cause (Gandon & Michalakis, 2000; de Roode *et al.*, 2008). For example, theoretical models have suggested that different host strategies including pathogen growth inhibition, infection avoidance, or tolerance, can affect whether pathogens evolve increasing, or decreasing virulence (Gandon & Michalakis, 2000; Restif & Koella, 2003; Miller *et al.*, 2005, 2006).

The overarching aim of this thesis is to explore how different host defense strategies influence host and pathogen evolution in North American house finches (*Heamorhous mexicanus*), and the bacterial pathogen *Mycoplasma gallisepticum*. The research herein utilizes a variety of techniques including mathematical modeling to generate hypotheses about the underlying evolutionary mechanisms and infection dynamics in this system, as well as analysis of the protective benefits of immune measures using transcriptomics and molecular methods. The consistent underlying feature throughout is the comparison of two host populations; Eastern finches from Alabama who have been exposed to *M.gallisepticum* for over 20 years, versus Western finches

from Tempe, Arizona, who have remained naïve to the disease (Staley et al. 2017, in press).

**The emergence of *M.gallisepticum* in North American house finches**

*M.gallisepticum* is a common pathogen of poultry (Avakiana & Leyb, 1993; Gaunson *et al.*, 2000a). In 1994, a novel strain emerged in North American house finches, first reported in Washington DC (Ley *et al.*, 1996; Fischer *et al.*, 1997), before spreading rapidly in the Eastern and Northern range of the species (Dhondt *et al.*, 2005). Infection in the house finch results in severe conjunctivitis and a disease of the upper respiratory tract (Ley *et al.*, 1996), resulting in extremely high mortality rates (Hochachka & Dhondt, 2000). In an effort to monitor disease spread, André Dhondt and colleagues set up the volunteer-based monitoring scheme, the House Finch Disease Survey (HFDS) (Dhondt *et al.*, 2006).

By the early 2000s, *M.gallisepticum* had moved Westwards, first appearing in those from Missoula, MT (Duckworth *et al.*, 2003) and later in Portland, OR (Ley *et al.*, 2006). Western populations are native to the U.S, differing genetically to Eastern populations with little known admixture (Hawley *et al.*, 2008). However the low density of Western house finches across the Great Plains, coupled with the non-migratory behaviour of the Western populations (Sauer *et al.*, 2012) meant that disease spread more slowly in these regions. Once established in different populations, it began to vary seasonally in prevalence on both local and regional scales (Hartup *et al.*, 1998; Altizer *et al.*, 2004; Dhondt *et al.*, 2005) characterised by semi-annual peaks,

corresponding to seasonal host aggregation and breeding (Hosseini *et al.*, 2004).

**Pathology and immune responses to *M.gallisepticum***

As aforementioned, *M.gallisepticum* manifests itself as a severe conjunctivitis, characterised by conjunctival edema and disease of the upper respiratory tract (Kollias *et al.*, 2004). Other symptoms of infection include decreased motor activity (Kollias *et al.*, 2004), mass loss (Bonneaud *et al.*, 2012a) and an increase in body temperature (Hawley *et al.*, 2012). In experimental infection studies, morbidity was high (100%;(Kollias *et al.*, 2004)), but mortality varied from just 5% (Kollias *et al.*, 2004) to over 90% (Farmer *et al.*, 2002).

As *M.gallisepticum* is an economically important disease affecting poultry (Stipkovits & Kempf, 1996), much of what we know about its pathogenicity and the immune processes involved with infection are from experimental infections of both virulent and attenuated strains of poultry MG in chickens (Mohammed *et al.*, 2007). MG has the ability to misdirect some components of the inflammatory response, such that epithelial membranes on respiratory mucosa are disrupted, allowing increased adherence and multiplication in these tissues (Gaunson *et al.*, 2006). The ability of Mycoplasmas to both suppress and potentiate different aspects of host immunity makes it challenging to determine which processes that are under host versus pathogen control (Staley & Bonneaud, 2015).

One approach to teasing apart host versus pathogen control of immune processes in house finches is to make use of the comparison between unexposed Western and exposed Eastern populations. In an experimental infection of finches from both populations in 2007, Bonneaud and colleagues found that birds from exposed (Alabama) populations up-regulated genes associated with immunity, relative to finches from unexposed populations (Arizona) (Bonneaud *et al.*, 2011). Furthermore, birds from unexposed populations down-regulated immune genes at two weeks post infection, suggesting that unexposed birds were more susceptible to immunosuppression, consistent with the evolution of resistance in Eastern birds (Wang *et al.*, 2006; Bonneaud *et al.*, 2011).

In a later study, it was revealed that significant differences in splenic gene expression between populations as early as 3dpi could be detected, indicating that the spread of resistance involved a change in innate immune processes (Bonneaud *et al.*, 2012b). Unexposed birds down-regulated genes associated with immunity at 14dpi, but up-regulated complement-factor H gene, which is known to constrain the activity of the complement cascade. However, exposed birds were able to up-regulate genes involved with protective immunity, such as CD74, which is associated with the assembly of MHC class II associated molecules. Thus, it was concluded that gene expression patterns were consistent with the hypothesis that some components of the immune response were being suppressed in finches from Western populations, and that birds from Eastern populations had enhanced protective immunity by comparison (Bonneaud *et al.*, 2012b). However, in spite of an awareness of

some of the components of the house finch immune response to MG, their protective benefits, as well as the conditions leading to rapid selection on host resistance traits, remains to be elucidated.

## Thesis overview

All of the work herein focuses on the exposed and unexposed populations described above, aiming to examine the conditions that allowed for rapid host evolution, and the functional significance of inter-population differences in host immune response. Each chapter is written as a discrete manuscript, with an abstract, introduction, methods, results and discussion.

### Chapter 2: Quantitative host resistance drives the evolution of increased virulence in an emerging pathogen

Emergent infectious diseases can have a devastating impact on host populations and generate high selective pressures on both hosts and pathogens. As a consequence, rapid adaptations in host resistance and pathogen virulence are frequently observed following an initial severe outbreak. However, it is often unclear to what extent hosts will evolve to prevent infection (*qualitative resistance*) or limit its deleterious effects (*quantitative resistance*) and what the evolutionary repercussions will be for the pathogen. Previous studies on MG in house finches have indicated that the evolution of host resistance occurred rapidly, within just a few generations (Bonneaud *et al.*, 2011), accompanied by parallel patterns of increased pathogen virulence (Hawley *et al.*, 2013). Using a two strain, two host SIR-

based evolutionary modelling approach, we investigate the dynamical interplay between host resistance and pathogen virulence with a specific emphasis on the likely resistance trait underlying the observed shift in host phenotypes and evolution towards higher virulence.

**Chapter 3: Protective benefits of the immune response to *M.gallisepticum***

Various studies have highlighted how a history of exposure to a pathogen can result in adaptive changes in immune responses(Best & Kerr, 2000; Kerr & McFadden, 2002). However, when pathogens have the ability to manipulate host responses, it is often unclear as to whether specific components of immunity are protective, or damaging (Sansonetti & Di Santo, 2007). Furthermore, certain aspects of the host immune response are often assumed to be protective, despite little evidence to suggest that they have a direct role in pathogen control. Such is the case with circulating anti-*M.gallisepticum* antibodies (IgY), for which no direct protective benefit has yet been found in poultry (Noormohammadi *et al.*, 2002), and for which the benefit has not yet been tested in house finches. Using measures of host pathology, host condition, measures of antibody concentration and RNA-sequencing to uncover differential gene expression, I examine the underlying differences in the host response to *M.gallisepticum* infection between exposed and unexposed populations in relation to the likelihood of recovery, pathogen clearance and mortality. In doing so, I uncover how a history of pathogen exposure has shaped the response to infection and provide precursory

evidence for how resistant hosts are able to avoid the damaging effects of infection.

**Chapter 4: Discussion and future directions**

Finally, in chapter 4 I discuss my findings in the context of what is known about host-pathogen evolution in house finches with MG, as well as the potential wider implications of my research in relation to other emerging infectious diseases. I conclude by suggesting some future directions in this area.

# Chapter 2: Quantitative host resistance drives the evolution of increased virulence in an emerging pathogen

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## Abstract

Emergent infectious diseases can have a devastating impact on host populations. The high selective pressures on both the hosts and the pathogens frequently lead to rapid adaptations in host resistance and pathogen virulence following an initial outbreak. However, it is often unclear whether hosts will evolve to decrease infection-associated fitness costs through a form of resistance that either prevents the establishment of infection (*qualitative resistance*) or limits its deleterious effects, for e.g. through immune functioning (*quantitative resistance*). Equally, the evolutionary repercussions these resistance mechanisms have for the pathogen are largely unknown. Here we take advantage of the well-documented outbreak of the highly pathogenic bacteria *Mycoplasma gallisepticum* in North American house finches. Using an evolutionary modelling approach, we investigate the dynamical interplay between host resistance and pathogen virulence. With a specific emphasis on the evolved resistance trait, we demonstrate that the observed rapid shift in host phenotypes following the outbreak is suggestive of strong selection pressure to reduce infection-associated mortality. This in turn created the ecological conditions that selected for increased bacterial virulence. Our results thus suggest that quantitative host resistance, which inadvertently helps to maintain high levels of disease prevalence in the population, is the key factor underlying the evolutionary interactions in this natural host-pathogen system.

## Introduction

Antagonistic interactions between hosts and pathogens can give rise to intense selection pressures and trigger rapid evolutionary changes in both (Buckling & Rainey, 2002; Paterson *et al.*, 2010). This is particularly true in the context of novel disease outbreaks, in which potentially devastating impacts on the host population are expected to feed back to the pathogen through a rapidly changing host environment (Lively, 1989; Best & Kerr, 2000; Paterson *et al.*, 2010). When faced with high infection-associated fitness costs, hosts can evolve either to prevent the establishment of infection, referred to as *qualitative resistance*, or limit its deleterious effects, referred to as *quantitative resistance*, through immune function (Gandon & Michalakis, 2000; Sepil *et al.*, 2013). Quantitative resistance is a term used to describe a scenario where by all hosts can become infected with a parasite, but resistant hosts are harmed less through traits that reduce the performance of a parasite in resistant hosts; differing to tolerance, which reduces the fitness costs of infection (Koskela *et al.*, 2002). For example, quantitative resistance is conferred through specific MHC-supertypes associated with a reduction of parasitaemia (parasite load) in great reed warblers (*Acrocephalus arundinaceus*) (Westerdahl *et al.*, 2012) and great tits (*Parus major*) (Sepil *et al.*, 2013) infected with *Plasmodium* parasites. Evidence for the evolution of different resistance strategies has been observed in many species; however, whether hosts will evolve qualitative or quantitative resistance, particularly in response to an emerging disease, remains largely unknown (Gandon & Michalakis, 2000).

Due to the close ecological relationship between pathogens and their hosts, the type of host evolutionary responses to infection is expected to impact pathogen exploitation strategies and subsequent evolution of virulence (the reduction to host fitness during infection) (Read, 1994; Roy *et al.*, 2000). Previous theoretical studies that distinguish host responses to infection as either preventing or limiting pathogen exploitation through reduced susceptibility or immune function (resistance), or offsetting infection-associated fitness costs (tolerance), have yielded useful predictions as to when we might expect evolution towards a high or low virulence strategy (Roy *et al.*, 2000; Miller *et al.*, 2006; Best *et al.*, 2014). However, we still lack a full understanding of the consequences of different resistance mechanisms (i.e., qualitative *versus* quantitative) on the evolution of pathogen virulence, particularly in wild populations with recurring epidemics. As certain resistance alleles are expected to be advantageous only when the pathogen is present (Westerdahl *et al.*, 2012), cycles of disease prevalence are likely to effect host-pathogen evolutionary trajectories.

Pathogens are generally assumed to maximise their fitness (in terms of their basic reproductive number, $R_0$) by optimising the trade-off between transmission rate and infection duration. Therefore, optimal virulence is highly dependent on these two factors. For example, a high proportion of qualitatively resistant hosts in a population will decrease overall parasite prevalence, which limits transmission opportunities and has thus been predicted to select for lower virulence (Baalen, 1998; Gandon & Michalakis, 2000). On the other hand, quantitative resistance traits, which permit infections but decrease infection duration, for example through immune

activation, are expected to select for higher virulence (Baalen, 1998; Gandon & Michalakis, 2000; Gandon *et al.*, 2002). What these studies highlight is that evolution of host resistance and pathogen virulence are intricately linked and require further investigation.

The outbreak of the bacterium *Mycoplasma gallisepticum* in North American house finches (*Heamorhous mexicanus*) mid-1990s following a jump from poultry provides us with a unique opportunity to disentangle the evolutionary interplay between host resistance and pathogen virulence in a natural system. The ensuing epizootic of severe conjunctivitis led to the death of millions of house finches (Luttrell *et al.*, 1998; Hochachka & Dhondt, 2000; Kollias *et al.*, 2004; Dhondt *et al.*, 2005) and was followed by the rapid evolution of host resistance, which spread from ~20% to ~80% within 12 years following the outbreak (Bonneaud *et al.*, 2011; Adelman *et al.*, 2013). Experimental studies showed that house finches from disease-exposed populations displayed lower pathogen load following infection than finches from disease-unexposed populations, as well as gene expression changes in immune-associated tissue that were consistent with increased resistance to infection (Wang *et al.*, 2006; Bonneaud *et al.*, 2011, 2012b). The underlying mechanism of resistance and whether it confers protection from infection establishment and/or from infection-induced morbidity or mortality is still unknown, however. Furthermore, parallel changes in bacterial virulence were also observed, with virulence, estimated as the severity of clinical symptoms, increasing over the course of the epizootic (Hawley *et al.*, 2013). If and how the spread of host resistance contributed to increased virulence over time remains to be established.

Here we used a modelling approach to investigate the consequences of different types of host resistance on temporal changes in host evolution, disease prevalence and pathogen virulence using the *M. gallisepticum* - house finch disease system. Building on previous findings of rapid resistance evolution in this system, this approach enabled us to test hypotheses generated in previous theoretical studies (Gandon & Michalakis, 2000) to an important and well-characterised avian system. We investigate the effects of qualitative resistance traits that prevent infection versus quantitative resistance traits that either limit disease severity and thus transmissibility or simply reduce infection-associated mortality on resulting patterns of host resistance and parasite virulence evolution. Our results suggest that the observed spread of host resistance was the result of strong selection pressure to reduce *M. gallisepticum* induced mortality, which in turn provided the competitive advantage for more virulent bacteria.

## Methods

In order to investigate the evolutionary dynamics of host resistance and pathogen virulence, we developed a two-strain, two-phenotype *SIRS* model with seasonal forcing. We divided the host population, $N$ (which is not assumed to remain constant), into two broad categories, resistant ($N_r$) and non-resistant ($N_{nr}$) hosts, with resistant hosts assumed to carry a resistance trait offering lower susceptibility to infection, faster infection clearance rate or lower disease-associated mortality. Hosts can become infected with either a high virulence strain ($h$) or a low virulence strain ($l$) of *M. gallisepticum* and

transmission is frequency dependent (proportional to $S/N$). For simplicity, we assumed that upon recovery birds gain full but waning immunity against reinfection and any differences in pathogen load that may occur between resistant and non-resistant hosts does not affect their capacity to transmit. Interactions between strains, such as partial cross-immunity or super-infection, were not considered. The rate of change in the number of *susceptible* ($S_{nr,r}$), *infected* ($I_{nr,r}^{h,l}$) and *recovered* birds ($R_{nr,r}$) was given by the following set of differential equations:

$$\frac{dS_{nr}}{dt} = b(t)N_{nr} - \beta(t)\left(\left(I_r^l + I_{nr}^l\right) + \lambda_1\left(I_r^h + I_{nr}^h\right)\right)\frac{S_{nr}}{N} + \delta R_{nr} - \mu(t)S_{nr}$$

$$\frac{dS_r}{dt} = b(t)N_r - \rho_1\beta(t)\left(\left(I_r^l + I_{nr}^l\right) + \lambda_1\left(I_r^h + I_{nr}^h\right)\right)\frac{S_r}{N} + \delta R_r - \mu(t)S_r$$

$$\frac{dI_{nr}^l}{dt} = \beta(t)\left(I_r^l + I_{nr}^l\right)\frac{S_{nr}}{N} - \sigma I_{nr}^l - \zeta I_{nr}^l - \mu(t)I_{nr}^l$$

$$\frac{dI_{nr}^h}{dt} = \lambda_1\beta(t)\left(I_r^h + I_{nr}^h\right)\frac{S_{nr}}{N} - \lambda_2\sigma I_{nr}^h - \lambda_3\zeta I_{nr}^h - \mu(t)I_{nr}^h$$

$$\frac{dI_r^l}{dt} = \rho_1\beta(t)\left(I_r^l + I_{nr}^l\right)\frac{S_r}{N} - \rho_2\sigma I_r^l - \rho_3\zeta I_r^l - \mu(t)I_r^l$$

$$\frac{dI_r^h}{dt} = \rho_1\lambda_1\beta(t)\left(I_r^h + I_{nr}^h\right)\frac{S_r}{N} - \rho_2\lambda_2\sigma I_r^h - \rho_3\lambda_3\zeta I_r^h - \mu(t)I_r^h$$

$$\frac{dR_{nr}}{dt} = \sigma\left(I_{nr}^l + \lambda_2 I_{nr}^h\right) - \delta R_{nr} - \mu(t)R_{nr}$$

$$\frac{dR_r}{dt} = \rho_2\sigma\left(I_r^l + \lambda_2 I_r^h\right) - \delta R_r - \mu(t)R_r$$

where *b(t)* is the seasonal birth rate, $\beta(t)$ is the seasonal transmission coefficient, *σ* is the recovery rate, $\zeta$ is the disease-associated mortality rate, and $\mu(t)$ is the natural and season-dependent death rate. Supplementary Figure S1 illustrates this model as a flow diagram.

We used two sets of scaling factors to investigate the (independent) effect(s) of host resistance ($\rho_1, \rho_2, \rho_3$) and pathogen virulence ($\lambda_1, \lambda_2, \lambda_3$). For example, $\rho_2(\geq 1)$ describes the relative increase in the recovery rate for birds of the resistant phenotype, whereas $\lambda_1(\geq 1)$ describes the relative increase in the transmission rate of birds infected with the more virulent strain. Considering host susceptibility, recovery rate and mortality rate independently allows us to investigate the full spectrum of resistance, from purely *qualitative* ($\rho_1 < 1, \rho_2, \rho_3 = 1$, i.e. decrease susceptibility but equal infection length and mortality rate) to purely *quantitative* ($\rho_1 = 1, \rho_2 \geq 1, \rho_3 \leq 1$, i.e. equal susceptibility but either increase clearance and/or decreased mortality).

Seasonal changes in house finch demography and aggregation rates have previously been shown to be important in generating semi-annual cycles of *M. gallisepticum* prevalence (Altizer *et al.*, 2004; Hosseini *et al.*, 2004). We therefore incorporated seasonality into our model by using time-dependent birth, death and transmission rates (see Supplementary Figure S2). Birth rates, $b(t)$, peak in July / August, whereas mortality, $\mu(t)$, is highest during winter months (see Supplementary Figure S2). Driven by social aggregation during the mating season and the formation of winter foraging flocks, we assumed that transmission rates, $\beta(t)$, fluctuate semi-annually (Altizer *et al.*,

2004). The seasonal birth, mortality and transmission rates are given as follows:

$$b(t) = b_0\left(\sin\left((t-0.1)\pi\right)\right)^{k_b}$$

$$\mu(t) = \mu_0\left(0.4 + 0.6\ (\sin\left((t-0.5)\pi\right)\right)^{k_d}$$

$$\beta(t) = \beta_0(0.2 + 0.8\left(\left(\sin\left((t-0.35)\pi\right)\right)^{k_t} + \left(\sin\left((t+0.35)\pi\right)\right)^{k_t}\right)$$

with $b_0$, $\mu_0$ and $\beta_0$ denoting the peak birth, death and transmission rates, respectively. $k_b$ , $k_d$ and $k_t$ are (even-valued) shape parameters that determine the length of a season.

We initialised the model assuming a 20% background prevalence in host resistance (in line with empirical observations;(Bonneaud *et al.*, 2011)) and equal prevalence of high and low virulent strains of *M. gallisepticum*. Host recovery rate was set at 2 months (Bonneaud, unpublished) and the loss of immunity was set at 14 months (Kollias *et al.*, 2004). Table 1 provides a summary of the model's parameters.

## Table 1: Model Parameters.

| Parameter | Description | Value | Range |
|---|---|---|---|
| $\boldsymbol{\sigma}$ | recovery rate (per year) | 6 | [5,10] |
| $\boldsymbol{\delta}$ | rate of loss of immunity (per year) | 0.9 | [0.5,1.5] |
| $\boldsymbol{\zeta}$ | disease associated death rate (per year) | 3 | [2,4] |
| $k_t$ | shape parameter (transmission) | 20 | - |
| $k_b$ | shape parameter (birth) | 80 | - |
| $k_d$ | shape parameter (death) | 10 | - |
| $\boldsymbol{\beta_0}$ | max transmission coefficient | 48 | - |
| $b_0$ | max birth rate (per year) | 3.61 | - |
| $\boldsymbol{\mu}_0$ | max death rate (per year) | 0.3 | - |
| $\boldsymbol{\rho_1}$ | susceptibility scale factor (resistance) | 1 | [0,1] |
| $\boldsymbol{\rho_2}$ | recovery scale factor (resistance) | 1 | [1,2] |
| $\boldsymbol{\rho_3}$ | mortality scale factor (resistance) | 0.1 | [0,1] |
| $\boldsymbol{\lambda_1}$ | transmissibility scale factor (virulence) | 1.2 | [1,2] |
| $\boldsymbol{\lambda_2}$ | recovery scale factor (virulence) | 1 | [0,1] |
| $\boldsymbol{\lambda_3}$ | mortality scale factor (virulence) | 2 | [1,5] |

## Results

### (a) General model behaviour assuming quantitative resistance

First, we simulated our model considering all hosts as equally susceptible to becoming infected, but with resistant and non-resistant hosts differing in their infection-induced mortality rates. As shown in Figure 1, this model captured the observed qualitative dynamics in disease prevalence over time, with the

two distinct peaks in spring and autumn corresponding to seasonal increases in host population densities and aggregation, and hence in transmission opportunities (Hosseini *et al.*, 2004).

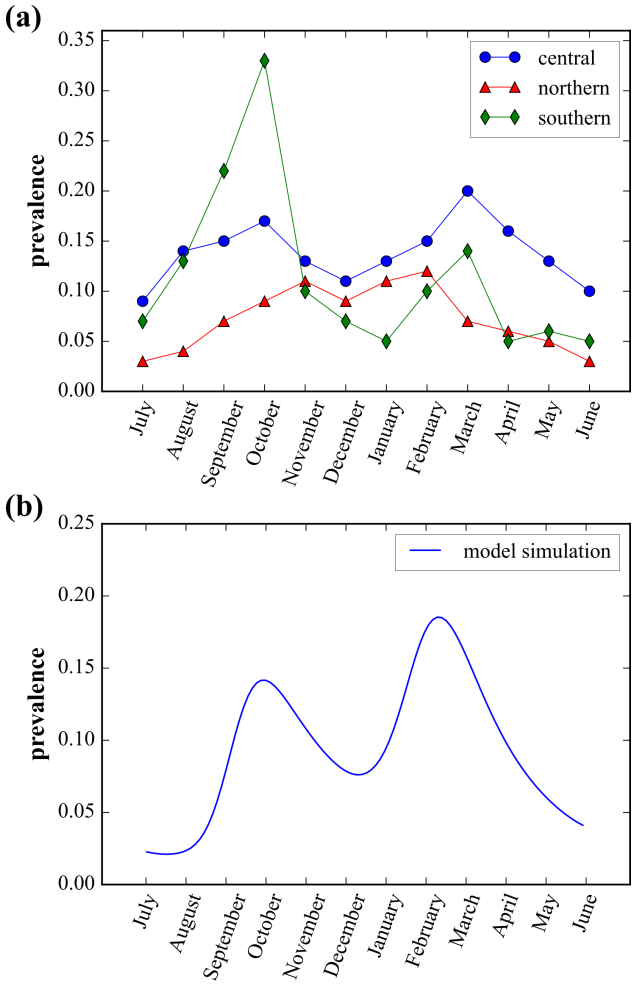


**Figure 1. Short-term dynamics of population level *M. gallisepticum* infection prevalence.** (a) Seasonal component of Empirical prevalence data adapted from (Altizer *et al.*, 2004; Hosseini *et al.*, 2004). Red line represents Southern regions of the Eastern United states, blue line represents central regions and green line represents northern regions. (b) Model simulation capturing the semi-annual dynamics driven by seasonal fluctuations in host densities and aggregation rates. Parameter values: $\boldsymbol{\rho_1 = 1, \rho_2 = 1, \rho_3 = 0.1, , \lambda_3 = 2}$; other parameter values as in Table 1.

Following the initial outbreak, we found that the number of susceptible hosts and the total host population size decreased significantly as a direct result of

602 high infection-associated mortality rates (Figure 2a). This decrease in the
603 number of susceptible hosts then led to a substantial decline in disease
604 prevalence (Figure 2b). When we stratified the host population into resistant
605 and non-resistant phenotypes (Figure 2c), our model revealed rapid
606 phenotypic changes in the host population in line with previous empirical
607 studies (Bonneaud *et al.*, 2011), with resistant hosts reaching ~80%
608 prevalence after around ten years post-emergence. In parallel, while the low
609 virulence pathogen strain dominated during the initial phase of the epizootic, it
610 became outcompeted by the more virulent strain after around 8 years (Figure
611 2b and d). These results therefore suggest that strong pathogen-induced
612 selection pressure and the subsequent increase in resistant host phenotypes
613 in the population created the conditions for a more virulent pathogen strain to
614 emerge and dominate (Figure 2d, S3).

615

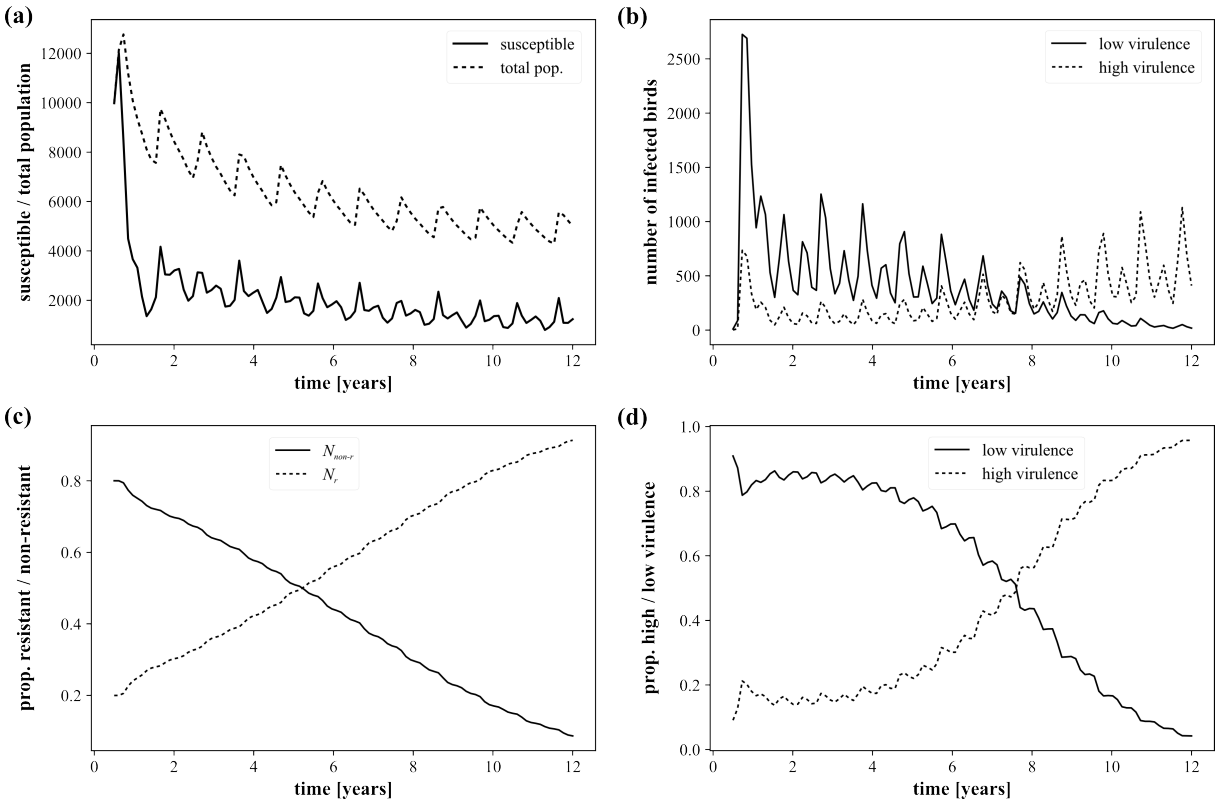


**Figure 2. Model simulations of host and pathogen population dynamics.** (a) Following disease emergence there is a significant decrease in the number of susceptible hosts (solid line) and total host population size (dashed line). (b) Number of individuals infected with the low virulence (solid line) and high virulence strain (dashed line) over time showing initial dominance of the low virulent strain, which eventually gets outcompeted. (c) Change in the proportion of the host population with a non-resistant or resistant phenotype ($N_{nr}$ and $Nr$, respectively). (d) Proportion of host population infected with low or high virulence strains over time showing an initial expansion of the low virulence strain until the number of resistant hosts has reached a critical threshold that tips the balance in favour of the more virulent strain. Parameter values: $\boldsymbol{\rho_1 = 1, \rho_2 = 1, \rho_3 = 0.1, \lambda_3 = 2}$; other parameter values as in Table 1.

Similar model behaviour was obtained when assuming quantitative resistance as an increase in recovery rate, which equally selected for host resistance in the population, albeit at a much-reduced rate (Supplementary Figure S4). What these results suggest is that disease prevalence needs to be maintained at a sufficiently high level for the fast and continued selection of host resistance traits. That is, both a reduction in susceptibility and a reduction in

636 the infectious periods drastically reduce the number of susceptible hosts and
637 hence disease transmission, which consequently lowers the selection
638 pressure in the population.

639

640 **(b) Qualitative resistance reduces the speed of phenotypic change**

641 We next tested the effects of different resistance strategies on the host's
642 evolutionary response to infection. As shown in Figure 2c and 3a (top graph),
643 quantitative resistance in the form of a reduction in disease-associated
644 mortality causes a rapid change in host phenotype distribution, with resistant
645 birds increasing in frequency from ~20% to ~80% in just ten years in line with
646 empirical observations. Instead, when modelling resistance as a difference in
647 susceptibility to becoming infected, we found a marked reduction in the speed
648 of resistance evolution, with resistant hosts only making up about half of the
649 population ten years after disease emergence (Figure 3a, bottom graph).
650 Running a sensitivity analysis over these two resistance traits (relative
651 susceptibility, $\rho_1$, and relative mortality, $\rho_3$) confirmed that resistant birds need
652 to have similar rates of infection, but a much reduced risk of mortality from
653 disease related causes, in order to explain the observed shift in host
654 phenotypes (Figure 3b).

655

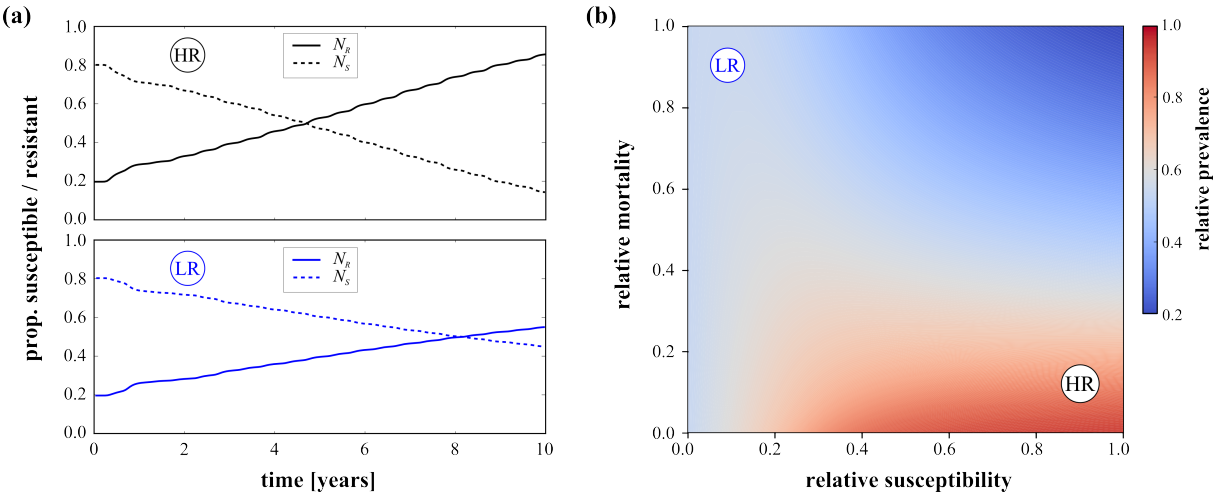


**Figure 3. Effect of relative susceptibility and mortality on selection of host resistance phenotype.** (a) Model output for the frequency of resistant (solid line) versus non-resistant (dashed line) host phenotypes over time. Reduction of infection-associated mortality leads to the rapid selection of resistant phenotypes (HR, top graph), whereas a reduction of susceptibility to infection slows down the selection for resistance (LR, bottom graph). (b) The effect of relative mortality and relative susceptibility of resistant birds on the prevalence of the resistant phenotype at ten years post emergence. Low frequencies of resistant phenotypes occur when mortality is similar between the two host phenotypes and when resistant hosts are significantly less susceptible to infection (LR). High levels of population-level resistance occur when resistant and non-resistant hosts are equally likely to become infected but experience significantly lower mortality (HR). Parameter values: $\boldsymbol{\rho_2 = 1, \lambda_3 = 2}$; other parameter values as in Table 1, except in (a): $\boldsymbol{\rho_1 = 0.9, \rho_3 = 0.1}$ (top) and $\boldsymbol{\rho_1 = 0.1, \rho_3 = 0.9}$ (bottom).

## (c) Host resistance and its effect on virulence evolution

Finally, we considered the selective impact of host resistance on the evolution of pathogen virulence. As demonstrated above (Figure 2b and d) and in line with empirical observations, assuming quantitative resistance as a decrease in mortality can cause the selection of more virulent bacteria over time. However, the exact conditions that favour a more virulent strain crucially depend on its mortality rate relative to that of a less virulent strain, $\lambda_3$and the extent to which infection-induced mortality is reduced in resistant hosts, $\rho_3$. To

681 demonstrate this we ran a sensitive analysis over these two scaling factors
682 ($\lambda_3$ and $\rho_3$) and recorded the relative frequency of both strains over time
683 (Figure 4). As shown in Figure 4a, under the assumption that disease-
684 associated mortality is much higher for the more virulent strain, selection will
685 favour the less virulent strain as the gain in transmissibility is outweighed by
686 the rapid loss of infected hosts. On the other hand, if the mortality rates from
687 infection with either strain are not too dissimilar and resistant hosts are
688 significantly more protected against infection-induced mortality, more virulent
689 bacteria will have the fitness advantage.

690 Crucially, whilst the other two resistance strategies (reduced susceptibility or
691 reduced infection length through faster recovery) can also select for increased
692 virulence (Supplementary Figures S5 and S6, respectively), the conditions
693 under which this may take place are such that either the disease and/or host
694 population goes extinct, or the rate of change in host phenotype distribution is
695 too slow to be compatible with the data. Taken together, our results thus
696 suggest that quantitative resistance, which offers the host protection against
697 disease associated mortality, was the most likely factor underlying the
698 observed co-evolutionary dynamics in this wildlife disease system.

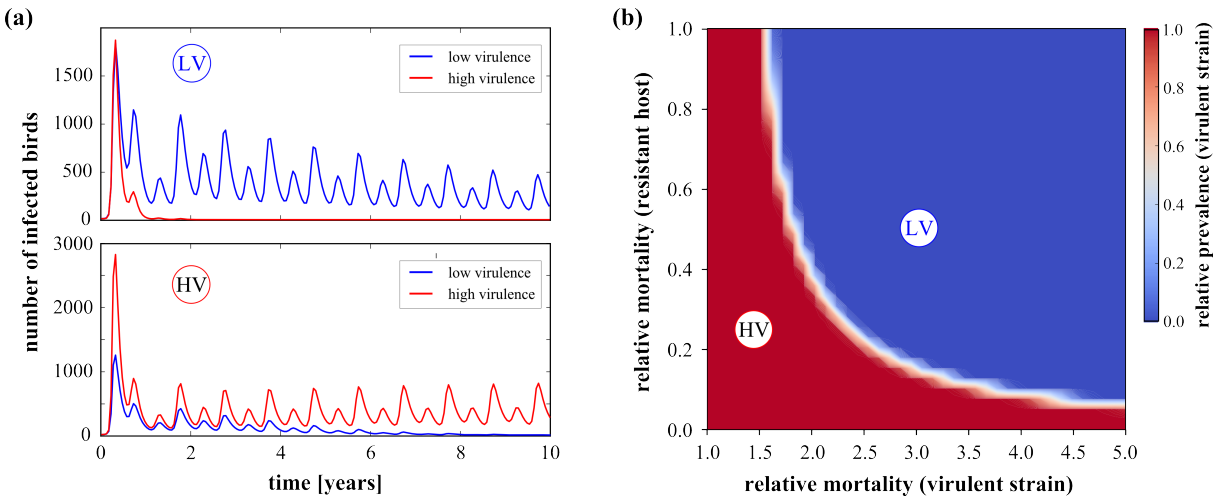


**Figure 4. Effects of host resistance on pathogen virulence evolution.** (a) Model output showing the number of birds infected with the low (blue) and high (red lines) virulence strain of *M. gallisepticum*. When resistant and non-resistant host phenotypes experience the same levels of infection-associated mortality the more virulent strain will go extinct (top graph), whereas greatly reduced infection associated death rates in resistant hosts can provide a fitness advantage for more virulent bacteria (bottom graph). (b) The effect of relative mortality caused by the high virulence strain versus the relative mortality in resistant hosts on the evolution of virulence, determined as the frequency of the high virulent strain at ten years post emergence. Parameter values: $\boldsymbol{\rho_1 = 1, \rho_2 = 1}$; other parameter values as in Table 1 as in Table 1, except in (a): $\boldsymbol{\lambda_3 = 3, \rho_3 = 0.5}$ (top) and $\boldsymbol{\lambda_3 = 2, \rho_3 = 0.1}$ (bottom).

## Discussion

In the present study, we identified the set of conditions that could explain the rapid selection of host resistance and parallel increased pathogen virulence following the epizootic outbreak of *M. gallisepticum* in North American house finches. Specifically, we have demonstrated that neither a reduction in host susceptibility to *M. gallisepticum* infections, nor an increase in parasite clearance rates, is compatible with the empirical data. Indeed, the impact of each on infection prevalence would cause a reduction in disease-induced selection pressure to the extent that we would no longer be able to observe shifts in both host and pathogen phenotypes. Instead, our results suggest that

723 the rapid, disease-induced selection of host resistance traits based on
724 reducing infection-associated mortality, for instance through a lowering of
725 pathogen load, was the key driver for the subsequent increase in bacterial
726 virulence in this important host-pathogen system.

727 Our results are in line with both empirical observations and theoretical
728 predictions. Experimental work on the evolution of resistance in house finches
729 has shown that resistance spread rapidly from standing genetic variation in
730 less than 12 years of disease exposure (Farmer *et al.*, 2005; Hawley *et al.*,
731 2007; Bonneaud *et al.*, 2011). This speed of host adaptation suggests that the
732 disease must have imposed a strong selection pressure on the host
733 population, a hypothesis further supported by the high rates of mortality
734 observed in the wild following outbreak (Hochachka & Dhondt, 2000). In
735 accordance, our modelling study demonstrates that for a phenotypic change
736 to occur in the host population, house finches must incur a high fitness cost
737 from being infected with *M. gallisepticum*, with non-resistant finches paying a
738 higher cost than resistant ones.

739 The results of our model would suggest that resistant finches not only
740 experience reduced mortality when infected, but that they should also display
741 a level of susceptibility to infection similar to that of non-resistant birds. When
742 host resistance is modelled as reduced susceptibility to infection, its negative
743 impact on population-level infection prevalence is indeed too great to maintain
744 the selection pressure that would account for the rapid change in host
745 phenotype frequencies. A similar outcome was obtained when modelling
746 resistance as increased recovery rate: selection pressure on host resistance

747 subsequently dropped, thereby slowing down the speed of host phenotypic
748 change. The most likely resistance trait under selection in this system
749 therefore consists of a reduction in infection severity, which is compatible with
750 the notion of quantitative resistance.

751 It has previously been proposed that qualitative resistance, which lowers a
752 host's susceptibility to infection, should select for decreased pathogen
753 virulence and that this effect should positively increase with the proportion of
754 resistant individuals in the host population (Gandon & Michalakis, 2000).
755 Although we also found some conditions under which reduced susceptibility
756 could lead to an increase in virulence, the parameter regions where this
757 occurred resulted in model behaviours that are incompatible with the
758 observed data, leading either to (host or pathogen) population extinction or to
759 a reduction in selection pressure precluding major shifts in host phenotype
760 frequencies.

761 A number of studies to date have focussed on characterising the immune
762 response of wild populations to emerging infectious diseases (Kerr &
763 McFadden, 2002; Gregory *et al.*, 2005; Bonneaud *et al.*, 2012b). The
764 canonical example of the parallel evolution of host resistance and pathogen
765 virulence following disease emergence is the eradication attempt of European
766 rabbits (*Oryctolagus cuniculus*) of Australia using the *Myxoma* virus. Following
767 the release of highly virulent strains in 1950, which resulted in a dramatic
768 decline of the rabbit population by over 99% (Marshall *et al.*, 1955), virulence
769 was found to decrease and resistance via enhanced innate immunity to
770 spread in the host population (Best & Kerr, 2000). Although these findings

771 suggest that quantitative resistance may have driven the evolution of
772 resistance in this case, genes underlying both qualitative and quantitative
773 forms of resistance can be found in the wild, suggesting a role of both in
774 shaping host-pathogen interactions and coevolution. For example, a study on
775 wild great tits showed that different super types of the same MHC gene can
776 confer either qualitative or quantitative resistance to avian malaria (Sepil *et*
777 *al.*, 2013). Determining which form of resistance is under selection will shed
778 light on the phenotypic change expected in the host population, as well as on
779 the pathogen's evolutionary trajectory (Gandon & Michalakis, 2000).

780 An important aspect influencing the long-term evolutionary outcome of host-
781 pathogen interactions are the costs associated with either form of resistance.
782 It has been proposed that resistance through protective immunity is expected
783 to evolve only when the cost of mounting the immune response is lower than
784 the cost of being infected (Antonovics & Thrall, 1994; Boots & Bowers, 2004;
785 Viney *et al.*, 2005). House finches from populations that evolved resistance
786 have previously been found to lose more body mass following experimental
787 infections with *M. gallisepticum* than finches from unexposed populations
788 (Bonneaud *et al.*, 2012a). The fact that resistance has spread despite this
789 indicates that the fitness benefit of resisting either infection or infection-
790 associated morbidity ultimately outweighs the shorter-term energetic cost of
791 resistance. Hence, while our model does not include costs associated with
792 resistance, we do not expect such costs to impact the results of this study
793 other than by influencing the probability that resistance will go to fixation and
794 that resistant phenotypes will decline in frequency once the disease goes
795 extinct.

In conclusion, our results reiterate the important influence that the mechanisms underlying host resistance can have on the mode and tempo of host phenotypic change and pathogen virulence evolution. Distinguishing between different forms of resistance in plants is common-place (Ewing *et al.*, 2000; Ramalingam *et al.*, 2003; Hein *et al.*, 2009), but has only scarcely been applied to animal or human disease systems despite its importance for public health. That is, many disease intervention measures that either prevent infections (e.g. vaccination) or enhance clearance (e.g. drug treatment) will effectively induce, or mimic, host resistance. Given the varying impacts of different forms of resistance on the evolution of pathogen virulence, inappropriate control measures can on occasion, result in undesirable outcomes, such as increases in pathogen virulence (Gandon *et al.*, 2001) and disease severity (Stevens *et al.*, 2007). Our study thus highlights the need for a more detailed investigation of the evolutionary host-pathogen interactions in order to minimise adverse effects of disease control.

# Chapter 3:Protective benefits of the immune response to *M.gallisepticum*

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## Abstract

The adaptive significance of immune components is now high priority on the research agenda of many ecologists and evolutionary biologists, because defining individual levels of host resistance is impossible without knowledge of the efficacy of immune measures in combating infection. Protective effects of immune measures are often assumed, although the extent to which they confer protection likely depends on the history of pathogen exposure, as well as the specific way in which the pathogen causes damage. Using RNA-seq based transcriptomics, we assessed differential expression of immune and immune associated genes between finches originating from unexposed and coevolved house finch populations to infection. In addition, we assess the efficacy of an easily measured immune component, systemic antibody (IgY) in protection from mortality, symptom severity and the likelihood of increased recovery or pathogen clearance. Stark inter-population differences in transcriptional profiles are demonstrated, consistent with exposed populations exhibiting an enhanced ability to avoid immune-pathological effects of infection. In addition, we find significantly higher IgY concentration in exposed, relative to unexposed, finch populations. However, we find no protective benefit to increased circulating IgY concentrations, suggesting the increased humoral response may be a by-product of the up-regulation of another immune component.

## Introduction

Infectious diseases exert profound selection pressure on their hosts; influencing many aspects of host biology from reproductive modes (Liersch & Schmid-Hempel, 1998) to behaviour (Berdoy *et al.*, 2000). Response to pathogen exposure includes a suite of combative defense mechanisms, which together make up the immune response. Evidence for the rapid evolution of these responses in wild populations is growing; from rabbits with *Myxoma* virus (Kerr & McFadden, 2002) to amphibians with chytridiomycosis (Savage & Zamudio, 2016). Formerly the exclusive domain of immunologists, the study of the adaptive significance of immune responses has become a high priority on the research agenda of many evolutionary ecologists, because the costs and benefits of the type and strength of response elicited strongly influence the fitness of the host (Sheldon & Verhulst, 1996). In spite of this, the characterization of immune responses of wild populations to emerging diseases is rare, largely due to a scarcity of techniques available to do so, particularly in non-model organisms.

One method for overcoming the lack of availability of reference genomes in non-model organisms is to use RNA-seq based transcriptomics (Grabherr *et al.*, 2013). However, this has scarcely been applied to wild systems, largely due to the inability to control conditions in wild populations and due to the potential for environmental contamination. However, this method is increasing in popularity in a range of taxa from birds (Videvall *et al.*, 2015) to amphibians (Campbell *et al.*, 2018). As hosts live in naturally varying environments, the extrapolation of laboratory results to wild populations is often tenuous or not

completely suitable (Pedersen & Babayan, 2011). Never the less, in spite of new techniques that are now available to characterize immune responses, it is often unclear as to whether individual immune measures are protective, or if they reflect individual levels of host resistance.

Often, antibody levels are used as a proxy for resistance in wild animals, due to the tractability of taking fecal or hematological samples in the field. Antibodies (also called Immunoglobulins), are produced by B lymphocytes in response to specific antigens (Janeway *et al.*, 2001) and act to control invading pathogens through various mechanisms, including neutralization (Mazanec *et al.*, 1992; Palladino *et al.*, 1995; Teoh *et al.*, 2012), prevention of adherence (Wallace *et al.*, 1991)or the activation of the complement cascade (Diebolder *et al.*, 2014). However, whilst the humoral response is expected to be protective, evidence suggests that antibodies may not always be effective against certain pathogens (Amorim *et al.*, 2016; Dumke & Jacobs, 2016), or that high antibody titres can be associated with more severe symptoms (Wells *et al.*, 2014). In many cases, it is also unclear to what extent levels of antibody correlate with levels of host resistance.

When diseases emerge in a novel host, it is possible to document how coevolutionary history of exposure changes the mechanisms by which hosts overcome infection. One such system is the bacterial pathogen *Mycoplasma gallisepticum* in North American house finches *(Haemorrhus mexicanus*). A common pathogen of poultry, it emerged in Eastern finch populations in 1994 (Ley *et al.*, 1996). In house finches, *M.gallisepticum* causes conjunctivitis and

911 a disease of the upper respiratory tract. When the pathogen first emerged in
912 finches, it resulted in the deaths of millions of birds (Hochachka & Dhondt,
913 2000). By the early 2000s, *M.gallisepticum* had spread to Western finch
914 populations, although epidemic spread was slowed over the Great Plains due
915 to relatively low house finch densities (Sauer *et al.*, 2012). In fact, some
916 populations, such as finches in Tempe, Arizona remain unexposed to this day
917 (Staley et al. 2017, in press). Changes to the dynamics of *M.gallisepticum*
918 prevalence in Eastern house finch populations to endemic levels has been
919 attributed in part to the rapid spread of host resistance, occurring within just
920 12 years (Bonneaud *et al.*, 2011, 2012b).
921
922 Much of what is known about the immune response to *M.gallisepticum* is from
923 the poultry literature. *M.gallisepticum* infection is associated with suppressing
924 certain aspects of immunity (Mohammed *et al.*, 2007) including T-cell activity
925 2 weeks post inoculation (Ganapathy & Bradbury, 2003), and has been shown
926 to induce inflammatory responses, thus increasing number of tissue lesions
927 (Ley, 2008). During the early stages of the epizootic in house finches, Eastern
928 birds from Alabama displayed transcriptional responses to infection that were
929 similar to those from unexposed, Western finches from Arizona. However, in
930 the years following, Eastern birds began to differ in their responses. Previous
931 studies have demonstrated that these individuals were able up-regulate genes
932 involved in innate and acquired immunity, and harbored lower conjunctival
933 pathogen loads (Bonneaud *et al.*, 2011, 2012b). These results indicated that
934 differences in transcriptional response between populations did not simply
935 arise through ecological differences, but rather accompanied the evolution of

936 resistance. However, the efficacy of innate and acquired immune responses
937 in conferring survival and recovery still remain to be elucidated.
938
939 To examine the efficacy of innate and acquired responses in fighting
940 *M.gallisepticum* infection, we conducted an experimental infection and
941 examined systemic antibody (IgY) concentrations in relation to recovery from
942 symptoms (measured by eye-lesion severity), pathogen clearance (measured
943 by PCR-detection of the pathogen) and mortality. Although local antibody
944 concentration has been previously shown to have a role in mediating the
945 control of *M.gallisepticum* infection in chickens (Gaunson *et al.*, 2006), no
946 such protective role of local or circulating antibodies has yet been established
947 in house finches. We next examined whole-blood gene expression profiles, as
948 blood has recently been shown to be a useful tissue in detecting immune
949 stimulation in passerine birds (Meitern *et al.*, 2014). By comparing birds from
950 Eastern (Alabama) and Western (Arizona) populations, we investigate how a
951 history of disease exposure has shaped the response to infection in Eastern
952 birds.
953
954 We make several broad predictions with regards to population differences in
955 immune response, and the potential protective benefits. First, our results
956 would suggest a potential, protective benefit of circulating antibodies if: i)
957 antibody concentration is significantly higher in finches originating from
958 Alabama at one or all of the time points measured (if this occurs at a time
959 point early-on in infection, this may be suggestive of an enhanced speed of
960 antibody production), or ii) increased antibody responses are associated with

decreased mortality, increased recovery from symptoms or increased pathogen clearance. Furthermore, if differences in transcriptional profiles between populations show finches from Alabama decreasing the inflammatory response, or up-regulating genes associated with adaptive immunity, this will suggest that avoidance of immune-manipulation by *M.gallisepticum* has played a role in the evolution of host resistance.

**Methods**

During the summer of 2015, wild house finches were captured from populations with no prior exposure to *M. gallisepticum* in Arizona (N=25; 13 males and 12 females) and from populations that have been exposed to *M.gallisepticum* since disease outbreak in Alabama (N=25; 12 males and 13 females) (Bonneaud et al submitted). Upon capture, lack of past and current infection was verified using serum plate agglutination assay (Luttrell *et al.*, 1996) and PCR amplification of *M.gallisepticum* DNA in choanal swabs (Luttrell *et al.*, 1996; Roberts *et al.*, 2001). All birds were brought back to aviaries at Arizona State University, where they were housed for the remainder of the experiment.

Finches were infected with *M.gallisepticum* isolates from 2007 (N=16), 2011 (N=16) and 2015 (N=18), with each isolate inoculated into 1 finch from Alabama and 1 finch from Arizona (Bonneaud et al submitted). Development of clinical symptoms was monitored by visually scoring eye lesion severity at both eyes (0–5 scale; (Kollias *et al.*, 2004)) at 3, 6, 8, 14, 21, 25, 28 and 34 days post-infection (dpi). Peak symptom severity was determined as the

maximum score reached over the course of the experiment. Birds that developed clinical symptoms of less than 4 (see below for rationale) and then returned to a symptom score of 0 during the course of the experiment were classified as having recovered.

Clearance of *M. gallisepticum* was determined from conjunctival and tracheal swabs taken at 8, 14, 21 and 28 dpi and stored in 600 µL of RNA*later* ® (Thermofisher Scientific) at -80°C; DNA was extracted from the swabs using Qiagen DNeasy Blood and Tissue kit (Tardy et al in prep). Amplification of *M. gallisepticum* DNA was performed on the DNA extracts from all swabs using *mgc2* primers developed by (García *et al.*, 2005). Birds were categorized as having cleared the infection if the swabs became negative during the experiment.

At 14dpi, ~250 µL of whole blood was taken by brachial venipuncture. An aliquot of blood was directly stored in Tempus™ Blood RNA tubes (Thermofisher Scientific, Leicester, UK) at -80°C for subsequent RNA extraction. We isolated serum from a second aliquot for ELISA through centrifugation at 2000XG for 15 minutes at 4°C; serum was stored at -80°C.

Host mortality was estimated based on the development of symptoms of scores 4 or 5, as these would lead to blindness-associated death in the wild (Bonneaud et al submitted). Therefore, when individuals reached peak symptoms of scores 4-5, this was described as a mortality event. The experiment was stopped at 35 dpi and all birds were euthanized as stipulated

by home office licensing. Protocols were approved by Institutional Animal Care and Use Committees (IACUC) of Auburn University (permit # PRN 2015-2721) and of Arizona State University (permit #15-1438R), and by Institutional Biological Use Authorizations to Auburn University (# BUA 500).

**ELISA**

Goat Polyclonal Passerine IgY-heavy and light chain Antibody (Bethyl laboratories, Inc, TX, USA) was diluted to 1:1000 dilution according to manufacturer protocol. The prepared antibody was then conjugated to a visible proprietary HRP (horse radish peroxidase) ligand for visualization in enzyme-linked immunosorbent assay (ELISA), according to instructions accompanying the Lighting-Link® HRP Conjugation Kit (Innova biosciences, Cambridge, UK) for an antibody: HRP molar ratio of 1:4.

*M.gallisepticum* specific antibody detection was performed with ELISA, with methods adapted from (Fassbinder-Orth *et al.*, 2016). Finch serum samples were diluted to 1:2400 in 1X sample conjugate diluent (Affinitech LTD, AR, USA) and 100 µL of each sample was added to plate microwells bound with inactivated *M.gallisepticum* antigen (Affinitech LTD). A standard curve of pooled sample was run on each plate in a 2-fold dilution series from 1:200-1:6400. Samples were run in duplicate, and care was taken to ensure that duplicates were distant from one-another to reduce variation caused by evaporation and edge effects. Plates were washed three times in 1X wash buffer (Bethyl laboratories, Inc), before adding 100 µL of the prepared HRP-conjugated Passerine IgY-heavy and light chain antibody, diluted to a

1:10,000 concentration with sample conjugant diluent (Bethyl laboratories, Inc). After incubation for one hour in darkness, the plate was washed three times and 100 µL of TMB one component HRP microwell substrate (Bethyl laboratories, Inc) was added to each well and incubated for 15 minutes in darkness. The reaction was stopped with 100 µL of 0.18M $H_2SO_4$ and the plate was read on a plate reader at 450nm within 30 minutes of stopping the reaction.

**RNA isolation, cDNA library preparation and sequencing**

Total RNA for ten birds infected with 2015 isolates of *M.gallisepticum* was extracted by a guanidinium thiocyanate-phenol-chloroform method using TRIzol® (Life Technologies, CA, USA). 100 µL of each whole blood sample was lysed and homogenized in 1ml of TRIzol® by a 20 second bead ruptor with silicone beads, before continuing with the standard TRIzol® protocol. RNA was subsequently purified using the RNeasy Micro-Kit (Qiagen), according to the manufacturer protocol, including DNase treatment and subsequent spin-column clean-up. The quality and RNA concentrations of each sample were determined by nano-drop (Thermofisher scientific), followed by Qubit (Life Technologies). Final RNA concentrations of samples were measured on a Bioanalyzer (Agilent, Manchester, UK), and checked for an RNA integrity number (RIN) of >8.

After quality and quantity checks, library preparation and sequencing was performed by Exeter University sequencing service. The libraries were prepared from 500ng total RNA using the TruSeq stranded mRNA sample preparation kit (Illumina Inc, Essex, UK) including poly-A selection.

Transcriptome sequencing was performed on the Illumina HiSeq2500 platform, using a 125 base pairs sequencing run generating 458.5 million paired-end reads.

***De novo* transcriptome assembly**

Raw read quality was determined using FastQC software v0.11.6 (https://www.bioinformatics.babraham.ac.uk/projects/download.html#fastqc). Adapters from each sample were removed individually using TrimGalore! v0.4.4, which removed poor-quality reads from the 3' end of the sequences and subsequently looked for and removed the standard Illumina adapters (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). The trimmed reads across all ten samples were then combined into a single database. Trinity v2.5.1 was run in strand specific (RF) mode to generate three assemblies, of which the best was selected through computing the N50 length statistics and proportion of reads mapping to the assembly (Honaas *et al.*, 2016).

**Transcript abundance estimation and gene expression analysis**

Results of the *de novo* transcriptome assembly were used to perform expression analysis for each sample, due to the lack of a fully assembled *H.mexicanus* reference genome. Tools embedded in the Trinity differential expression module were used to do so, including Bowtie2 for mapping reads against the reference transcriptome and abundance estimation calculation using RSEM. Differential expression levels between unexposed (Arizona) and adapted (Alabama) were determined using the RSEM read counts matrix,

alongside the Bioconductor edgeR package. Only the isoforms represented with at least a four-fold change and an adjusted P-value (FDR) of 0.01 were considered as differentially expressed in the comparison between individuals from Eastern and Western populations.

**Functional annotation of the transcriptome**

The functional annotation of each predicted differentially expressed protein-coding sequence was performed with a search against the nonredundant (NR) database on NCBI using BLAST (http://www.blast2go.com) with an *E*-value threshold of 1e-3 (Conesa *et al.*, 2005). Blast2GO was also used to search through the NR database and assign Gene Ontology (GO) terms for all differentially expressed transcripts. GO biological processes were also assigned to each transcript through manually searching the Uniprot database.

We found a total of 138 differentially expressed candidate transcripts that were differentially expressed with a log-fold change of >4 between populations ($P<0.01$). The significance of differences in expression of these transcripts was confirmed by the Benjamini and Hochberg FDR corrections ($FDR<0.01$). Of these, 103 showed high similarity to known vertebrate homologues or hypothetical protein sequences in other species (*E*-value cut-off of 1e-3; Table S7). The remaining 35 transcripts were not successfully matched to known protein sequences.

The aim of our study was to test whether there is differential expression of genes directly linked to the response to infection between populations.

Previous studies have demonstrated that there are energetic costs associated with immune responses (Bonneaud *et al.*, 2012a), as well as differences in the expression of immune-associated genes in spleen tissue (Bonneaud *et al.*, 2011, 2012b). Therefore, we further processed these 103 annotated transcripts by carrying out literature searches to identify 20 specific transcripts associated with immune function, angiogenesis, autophagy, glucose metabolism, inflammation, mitochondrial electron transport chain, proteolysis and stress/damage, to assess underlying differences in response to infection, attributable to differences in history of pathogen exposure (Table 2).

**Statistical analyses**

All statistical analyses were conducted using RStudio v0.99.902 (R Studio team, 2017). Six individuals exhibited vastly higher antibody concentration at day 14 and 28, and deviated drastically from other individuals. Analyses were first run including those individuals and secondly with excluding them as outliers. Significance of results did not differ after their removal, so they were excluded from the data set for all analyses, leaving 44 individuals. General linear mixed effect models (Gaussian family, lme4 package) were used to investigate the effect of population of origin and days post inoculation (dpi) on antibody concentration (Table S1). Bacterial isolate ID and year of *M.gallisepticum* isolate were controlled for as random effect terms. We then tested for interactions between antibody concentrations at specific time points between populations using paired t-tests, with a significance level of 0.05.

To assess if circulating IgY was associated with symptom severity, we removed individuals who did not develop symptoms (n=6). We then used a linear regression with mean quantitative conjunctival score as the response variable and host IgY concentration at 14, 28 and 35 dpi as the explanatory terms.

Second, we investigated the effect of host IgY concentration on the probability of developing clinical symptoms that would be lethal in the wild using a logistic regression with mortality as the response term and host IgY concentration at 14, 28 and 35 dpi as the explanatory terms. Next, the relationship between host IgY and pathogen clearance was examined using logistic regression with clearance as a response variable and host IgY concentration at 14 and 28 as explanatory terms. Clearance was defined using qualitative symptom scoring, which was measured until day 34. We therefore excluded antibody concentration at 35dpi from our clearance models. Logistic regression was also used to test the association between the response term clearance and the explanatory terms host IgY concentration at 14 and 28. Measurements of symptom score (and therefore host recovery) were taken until 34 dpi, and so antibody concentration at 35dpi was excluded from the analyses. For all models with a single explanatory term significance was determined by comparing the MAM with the null model (which only contained the response variable) using ANOVA.

## Results

### Antibody Response

#### 1. Population differences in concentration of systemic IgY

To identify whether an increased humoral response had accompanied the evolution of resistance in coevolved house finch populations, we compared systemic IgY concentration between individuals originating from coevolved and unexposed populations at 14, 28 and 35 days post inoculation (dpi). Alabama birds mounted a higher antibody response over the course of infection than Arizona birds (GLM, estimate=0.01, $X^2$=8.99, p=<0.01, Table S1). However, this population difference was due to differences in antibody production at day 14 only (14 dpi: paired t-test, $T_{19}$= 2.15, P=0.04; 28 dpi: paired t-test, $T_{19}$= -0.83, P= 0.42; 35 dpi: paired t-test, $T_{19}$= -1.72, P= 0.10).

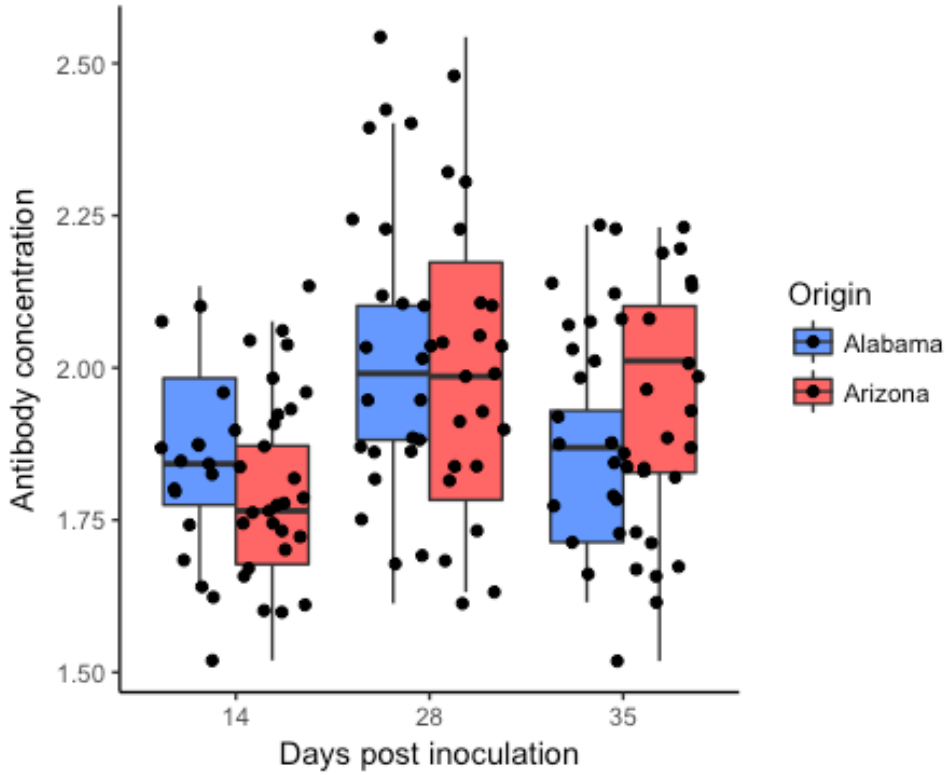



**Figure 1. Systemic antibody (IgY) concentration in finches from coevolved, Alabama (blue) and unexposed, Arizona populations (red) at 14, 28 and 35 dpi.** Populations differ significantly in their early IgY responses two weeks post inoculation.

## 2. Protective benefits of IgY

Mean symptom severity was not associated with circulating antibody concentration at day 14 ($R^2$ =0.01688, $t_{42}$ = -0.849, P = 0.4005; Y=72.63 - 15.11X), day 28 ($R^2$ =0.0006128, $t_{42}$ =-0.16, P = 0.8733; Y= 48.744 -1.808X) or day 35 ($R^2$ = 0.06568, $t_{42}$ = 1.718, P = 0.0931; Y= 0.3712 23.3488 X), indicating that antibody levels had no significant relationship with infection associated damage to host conjunctival tissues. Similarly, we found no significant association between antibody concentration at day 14 (logistic regression: Z= -1.534, df=42, p=0.1128), 28 (logistic regression: Z= 0.557,

df=42, p= 0.5753) or 35 (logistic regression: Z= 1.192, df=42, p= 0.2248) on the probability of host death, suggesting that antibody concentration had no relationship with the log likelihood of infection induced mortality. Furthermore, we found no significant association between IgY levels at days 14 (logistic regression: Z= 1.469, df=40, p= 0.1245) or 28 (logistic regression: Z= 0.228, df=40, p= 0.8206) and the probability of recovery from symptoms. Finally, antibody concentration was not a significant predictor of the probability of pathogen clearance (IgY day 14: logistic regression: Z= 0.215, df=42, p=0.8305; IgY day 28: logistic regression: Z= -0.800, df=42, p= 0.4007), which indicates that antibody levels had no significant relationship with the probability of clearing infection.

**Population differences in gene expression**

The gene expression profile of birds from the unexposed population (Arizona) was characterised by the up-regulation of genes involved with innate immunity (Akrin-2 isoform X1, Lysophosphatidylcholine acyltransferase 1 and Sorting nexin-3 isoform X1); glucose metabolism via the insulin receptor pathway (Insulin receptor substrate 4 and Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha isoform); inflammation (Cyclin-dependent kinase 19 like); proteolysis (Mast-cell protease 1A-like and Dipeptidyl peptidase 2 isoform X3) and response to damage/stress (DNA-polymerase kappa isoform X1). However, the gene expression profile of birds originating from the coevolved population (Alabama) was characterised by the up-regulation of genes associated with adaptive immunity (AP-1 complex subunit

gamma-1; inflammation (NF-kappa-B-activating protein); angiogenesis (Heparanase-isoform X2); autophagy (Myotubularin-related protein 8 and Autophagy-related protein 9A) and the mitochondrial electron transport chain (Dynamin-1-like protein isoform X4; Calcium uptake protein 2, mitochondrial isoform X2 and NADH dehydrogenase [ubiquinone] flavoprotein 3, mitochondrial; Table 2, Figure 3).

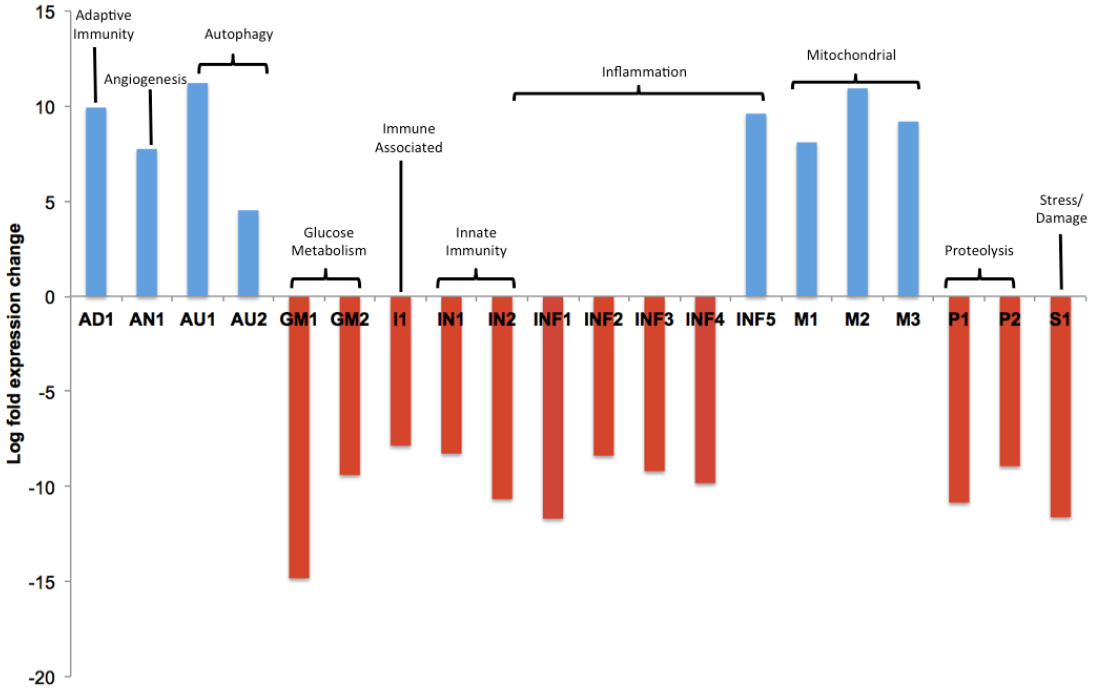



**Figure 2. Log fold difference in transcript expression.** Subset of transcripts associated with adaptive immunity (AD; N=1); angiogenesis (N=1); autophagy (AU; N2); glucose metabolism (GM; N=2); immunity (I; N=1); innate immunity (IN; N=2); inflammation (INF; N=5); mitochondrial electron transport chain (M; N=3); proteolysis (P; N=2) and stress/damage (S; N=1). Negative values represent higher expression in Arizona birds relative to Alabama and positive values represent higher expression in Alabama relative to Arizona. Abbreviations were assigned to correspond with grouping by function(Table 2).

**Table 2. Differentially expressed transcripts between unexposed and coevolved finch populations**

| Vertebrate Homologue | LFC | Abbreviation | GO Biological Process | Grouping |
|---|---|---|---|---|
| **AP-1 complex subunit gamma-1** | **9.92** | **AD1** | **Antigen processing and presentation of exogenous peptide antigen via MHC class II** | **Adaptive immune response** |
| **Heparanase isoform X2** | **7.75** | **AN1** | **Angiogenesis involved in wound healing** | **Angiogenesis** |
| **NF-kappa-B-activating protein** | **9.61** | **INF5** | **T cell differentiation in thymus** | **Adaptive immune response** |
| **Myotubularin-related protein 8** | **11.22** | **AU1** | **Negative regulation of autophagy** | **Autophagy** |
| **Autophagy-related protein 9A** | **4.53** | **AU2** | **Autophagosome assembly** | **Autophagy** |
| Insulin receptor substrate 4 | 14.83 | GM1 | Insulin receptor signalling pathway | Glucose metabolism |
| Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha isoform | 9.40 | GM2 | Insulin receptor substrate binding | Glucose metabolism |
| Ectonucleotide pyrophosphatase/phosphodiesterase family member 3 | 7.86 | I1 | Immune response | Immune associated |
| Akirin-2 isoform X1 | 9.83 | INF4 | Innate immune response | Innate immune response |
| Lysophosphatidylcholine acyltransferase 1 | 8.27 | IN2 | Neutrophil degranulation | Innate immune response |
| Sorting nexin-3 isoform X1 | 10.67 | IN3 | Intralumenal vesicle formation | Innate immune response |
| Cyclin-dependent kinase19-like | 11.69 | INF1 | Positive regulation of inflammatory response | Inflammation |
| **Dynamin-1-like protein isoform X4** | **8.10** | **M1** | **Mitochondrion organization** | **Mitochondrial** |
| **Calcium uptake protein 2, mitochondrial isoform X2** | **10.94** | **M2** | **Mitochondrial calcium ion homeostasis** | **Mitochondrial** |
| **NADH dehydrogenase [ubiquinone] flavoprotein 3, mitochondrial** | **9.19** | **M3** | **Mitochondrial ATP synthesis coupled electron transport** | **Mitochondrial** |
| Mast cell protease 1A-like | 10.85 | P1 | Proteolysis | Proteolysis |
| Dipeptidyl peptidase 2 isoform X3 | 8.94 | P2 | Proteolysis | Proteolysis |
| DNA polymerase kappa isoform X1 | 11.62 | S1 | Cellular response to DNA damage stimulus | Stress |
| Tyrosine-protein kinase Fer-like | 8.39 | INF2 | Actin cytoskeleton reorganization | Immune associated |
| E3 UFM1-protein ligase 1 | 9.20 | INF3 | Negative regulation of apoptotic process | Immune associated |

A subset (N=19) of the successfully annotated, differentially expressed isoforms (N=103) in Alabama (bold) and Arizona, chosen for their relation to immunity; inflammation; mitochondrial electron transport chain; proteolysis; stress; glucose metabolism and angiogenesis. For full details of differentially expressed isoforms see Supplementary Table 1.

## Discussion

Many parasites, including *M.gallisepticum* are capable of manipulating host immune responses. This means that assessing which components of host immune responses are protective can be complex. Here, we demonstrate that finches from coevolved populations show higher systemic IgY concentration relative to those from unexposed populations at 2 weeks post infection, but that IgY concentration has no clear relationship with host recovery, mortality or clearance of *M.gallisepticum*. Transcriptional profiles of both populations revealed stark inter-population differences, with finches originating from unexposed populations exhibiting greater expression of transcripts that relate to inflammation and innate immune processes relative to those from coevolved populations, who overall, showed enhanced adaptive immunity. Furthermore, our results suggest that coevolved birds do not benefit from protection through increased antibody response, and exhibit the potential to avoid immune manipulation by the pathogen.

Our results suggest that antibody concentration is significantly higher in finches originating from Alabama at 14 dpi. However, we were unable to find a link between IgY concentration and recovery, mortality and pathogen clearance. This may be attributable to the fact that we know relatively little about the role of IgY in pathogen control. IgY is understood to be the functional equivalent of mammalian IgG, which (among its versatile roles) is involved in antigen detection, although little is known about its role in activating complement pathways (Warr *et al.*, 1995). Previous studies in poultry have found no evidence for a protective role of IgY against

*M.gallisepticum* infection (Noormohammadi *et al.*, 2002). However, three independent studies of *M.gallisepticum* infection in chickens have suggested a primary role for the local secretory antibody, IgA in controlling *M.gallisepticum* infection through the prevention of bacterial establishment in mucosal cells at the site of infection (Yagihashi *et al.*, 1987, 1992; Avakiana & Leyb, 1993). However, given the tractability of taking haematological samples relative to tracheal washings in live birds, systemic IgY was examined here as an effective measure of the level host resistance through investigating its efficacy.

One reason for inter-population differences in antibody response may be that higher circulating antibody titres arise a result of increased activation of the other innate/acquired processes that are effective for protection. In concordance with results from a previous experimental infection of birds from the same populations (Bonneaud *et al.*, 2012b), we found transcriptional differences between populations in genes related to both innate and acquired immune components. Antibodies are generated by the action of the adaptive immune system, and therefore it is entirely plausible that heightened antibody concentration may be related to proliferation of B-lymphocytes in response to infection. It is also important to note that much of the variation in host immune phenotype is likely influenced by pathogen genotype x host genotype interactions (Lazzaro & Little, 2009). Indeed, house finches are continually adapting to *M.gallisepticum*, and therefore it is unlikely that their response to infection is optimally adapted at any one point in time.

One of the key mechanisms for pathogen clearance in vertebrate immunity is the inflammatory response (Ashley *et al.*, 2012). Dysregulation of this response can be damaging to host tissues (referred to as autoimmunity) or at worst, fatal (Warrington *et al.*, 2011; Wiersinga *et al.*, 2014). Recent studies in house finches have shown that the pathogenicity of *M.gallisepticum* involves the promotion of pro-inflammatory cytokines at the early stages of infection (3-6 dpi) (Vinkler *et al.*, 2018). Here we show that genes associated with promoting and regulating inflammation were highly expressed in Arizona finches. First, cyclin dependent kinase 19-like (INF1) was up regulated in finches from Arizona, relative to birds from Alabama. Cyclin dependent kinases (CDKs), are part of the pro-inflammatory response and expression of the components of the CDK/cyclin system are regulated by the NFκβ (nuclear factor κβ) transcription factors (Schmitz & Kracht, 2016). In addition, three other genes: Tyrosine-protein kinase Fer-like (INF2); E3 UFM1-protein ligase 1 (INF3) and Akirin-2 isoform X1 (INF4) were also more highly expressed in Arizona finches relative to Alabama. INF2 acts downstream of EGFR to promote activation of NFκβ and cell proliferation (Tsygankov, 2003), INF3 is thought to be involved with modulating NFκβ signalling (Li *et al.*, 2017), and INF4 is necessary for the expression of a subset of NFκβ genes, as well as IL-6 production (Ghosh & Hayden, 2008).

In finches from coevolved populations, we find only one gene with a potential link to inflammation: NFκβ activating protein (INF5), which induces pro-inflammatory NFκβ transcription factors (Baeuerle & Henkel, 1994). Evidence of a pro-inflammatory response in coevolved finches is consistent with recent

results from Vinkler and colleagues, who demonstrate strong pro-inflammatory responses in coevolved birds originating from Virginia in response to a post-resistance isolate of *M.gallisepticum* (Vinkler *et al.*, 2018). However, as birds originating from unexposed populations appear to show heightened expression of a higher number of genes relating to inflammation compared with those from coevolved populations, this may be indicative that coevolved birds have developed an enhanced ability to decrease their inflammatory responses to *M.gallisepticum*, which could protect individuals from immune manipulation by the pathogen.

Along side the potential for avoidance of immune-manipulation, finches from Alabama also appear to show enhanced adaptive immunity. Here, we show that finches originating from Alabama up-regulate AP-1 complex subunit gamma-1, which is associated with antigen presentation via MHC class II (Hiltbold & Roche, 2002). The major histocompatibility complex (MHC) is a component of the acquired immune response, involved in pathogen recognition. The role of MHC molecules is to bind to foreign antigens and present them on cell surfaces for recognition by T cells (Janeway *et al.*, 2001). In chickens, a shift in infiltrating populations of $CD4^+$ and $CD8^+$ T-cell receptors associated with MHC recognition has been recorded in response to infection with *M.gallisepticum* (Gaunson *et al.*, 2000b). In house finches, increased expression of MHC invariant chain II, which is essential for the assembly of MHC class II molecules, was also found in finches from Alabama, with down regulation of this gene in Arizona birds (Bonneaud *et al.*, 2012b),

suggesting that the MHC complex may have been subject to selection during the evolution of resistance.

Mounting a protective immune response is usually accompanied by some physiological cost (Boots & Haraguchi, 1999) and it has been known for some time that the strength of immune responses are dependent on trade offs with other energy-requiring processes, such as reproduction (Webster & Woolhouse, 1999). In a previous study, it was shown that finches originating from Alabama lost significantly more mass relative to those from Arizona in response to infection, and that these individuals also harbored lower bacterial loads, and highly expressed immune-related genes (Bonneaud *et al.*, 2012a), suggesting that infection may give rise to different energetic needs in birds from coevolved versus unexposed populations. In our study, finches from Alabama up-regulated three genes involved in the mitochondrial electron transport chain, and therefore associated with energy expenditure/usage: Dynamin-1-like protein isoform X4; Calcium uptake protein 2, mitochondrial isoform X2 and NADH dehydrogenase [ubiquinone] flavoprotein 3. Taken together, the results of the present study may provide further evidence for a distinct cost of immunity to *M.gallisepticum*, demonstrating that energy requirements during infection may differ between populations.

In addition, we were able to find evidence of another inter-population difference in energy usage that may relate directly either differential costs of infection, or may represent another mechanism of host manipulation by *M.gallisepticum*. Finches from unexposed populations showed significant up-

regulation of two genes involved in glucose metabolism via the insulin transduction pathway: Insulin receptor substrate 4 and Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha isoform, as well as a gene involved with the stress response: DNA polymerase kappa isoform X1. The insulin transduction pathway is essential for maintaining glucose homeostasis, although has many roles including countering inflammation through inhibition of NFκβ pathways (Dandona *et al.*, 2001). Studies examining physiological stress, have shown that the stress system and the immune system are intimately linked (Elenkov *et al.*, 1999), with periods of acute/chronic stress either boosting or decreasing the immune response respectively and resulting hypercortisolemia (Dhabhar *et al.*, 2012). Furthermore, as insulin is directly opposed by the action of cortisol, infection can sometimes lead to hyperglycemia through hyperinsulinemia or in extreme cases, insulin resistance (McGuinness, 2005). In the future, an additional comparison with a non-inoculated individuals is necessary in order to determine whether Alabama birds in fact down-regulate genes involved with insulin pathways relative to Arizona birds, in which case, this may purely represent differential energy usage between populations. Conversely, our results may demonstrate an interaction between stress and the action of the immune system in unexposed individuals if these genes are up regulated in Arizona birds only; in which case, this may indicate that coevolved populations have evolved a mechanism to avoid this damaging effect.

Through examining immune measures in relation to measures of host health, as well as using next generation sequencing techniques, we were able to uncover the lack of protective benefits of IgY, as well as elucidate some of the

other immune components that may confer host survival. A number of unexplored avenues remain. For example, a more comprehensive understanding of the pathogenesis and virulence factors of *M.gallisepticum* in house finches specifically will enable a more thorough understanding of the results presented here. In particular, this would help in partitioning which components of the immune system are host and pathogen driven. Furthermore, comparisons of responses to infection at additional time points during infection (for example in the early stages) and their relationship with host fitness are needed to further explore in detail, the adaptive significance of immune measures.

## Supplementary figures and tables

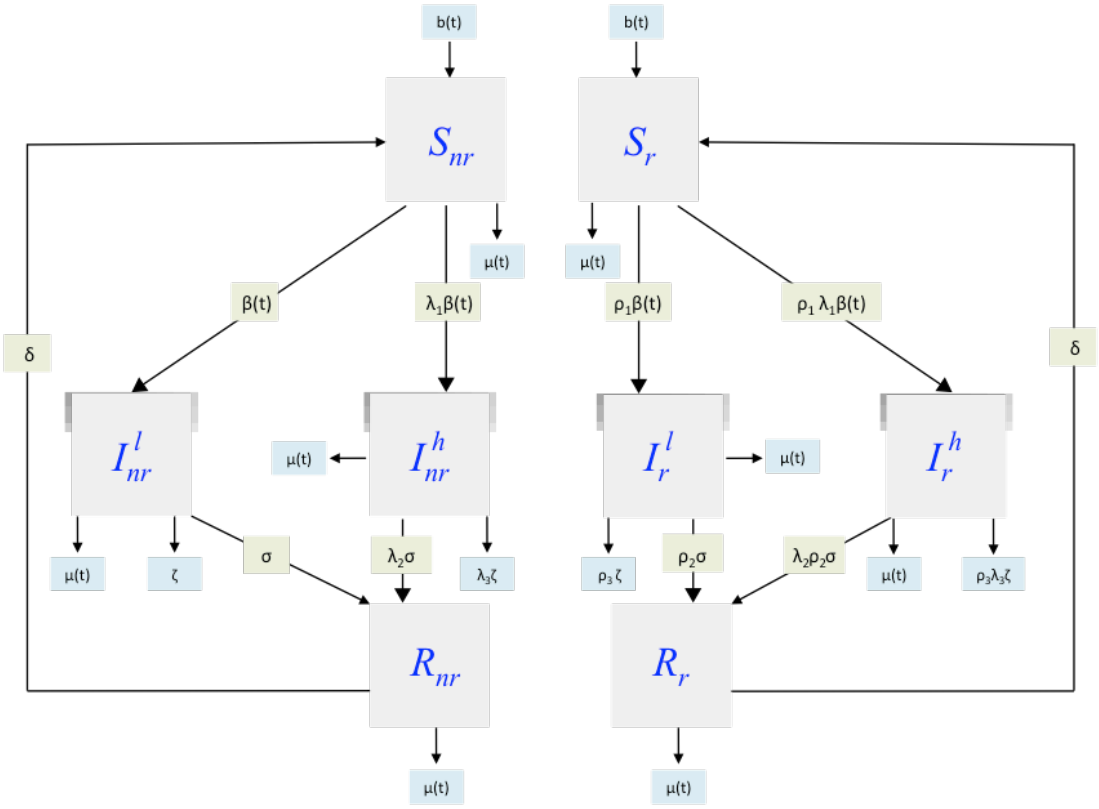



**Figure S1. Flow diagram of the 2 strain - 2 phenotype SIR model.** Model illustration showing the flow of movements of individuals in the population $(N_s + N_r)$ subdivided into $(N_s = S_s + I_s^l + I_s^h + R_s)$ and $(N_r = S_r + I_r^l + I_r^h + R_r)$ where *b(t)* is the seasonal birth rate, $\beta(t)$ is the seasonal transmission coefficient, $\sigma$ is the recovery rate, $\zeta$ is the disease-associated mortality rate, and $\mu(t)$ is the natural and season-dependent death rate.

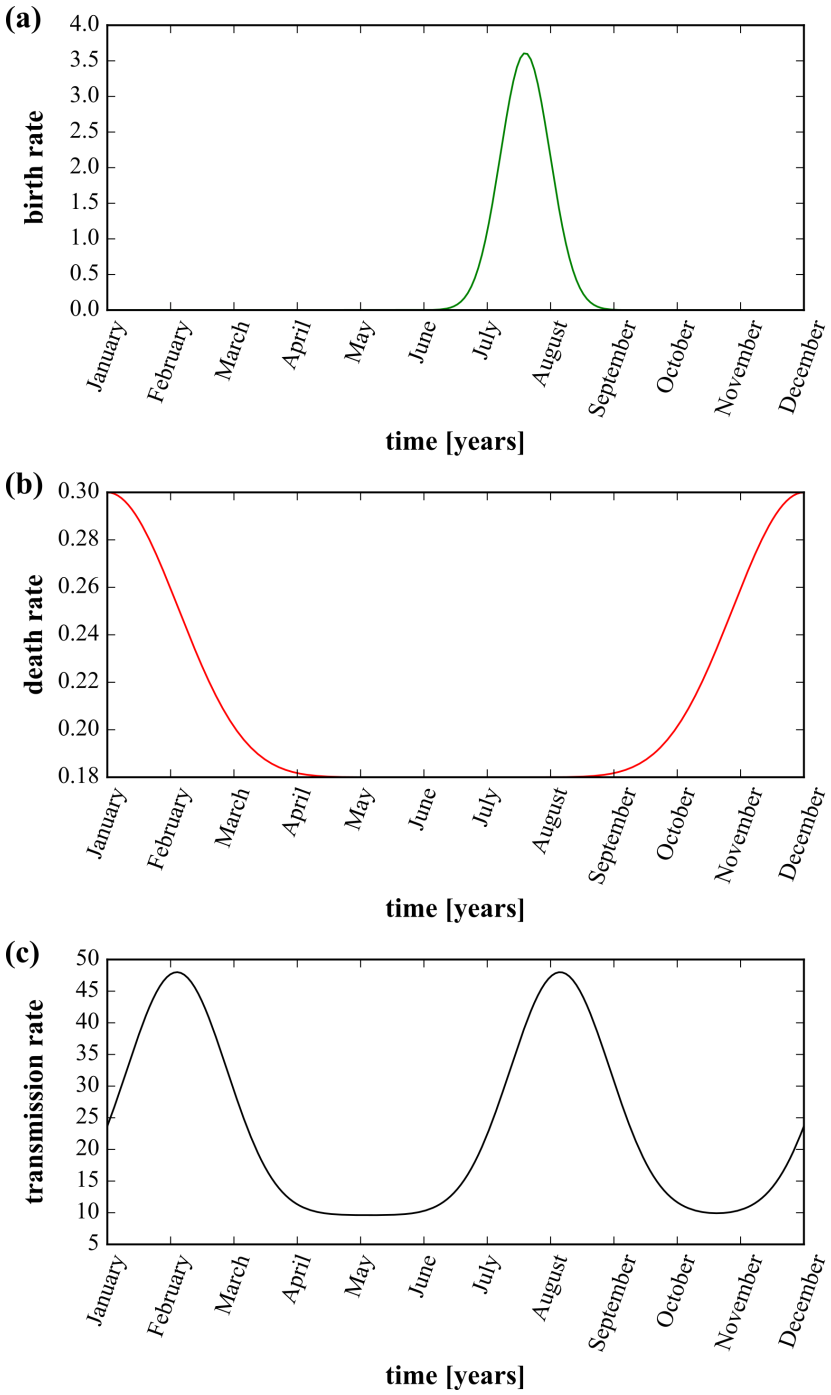



**Figure S2. Seasonal variation in birth, death and disease transmission.** (a) Seasonal variation in birth rate, $b(t, b_0, k_b)$, with peaks in mid-summer. (b) Seasonal variation in mortality rate, $\mu(t, \mu_0, k_d)$. (c) Semi-annual variation in disease transmission rate, $\beta(t, \beta_0, k_t)$. Parameter values as in Table 1 (main text).

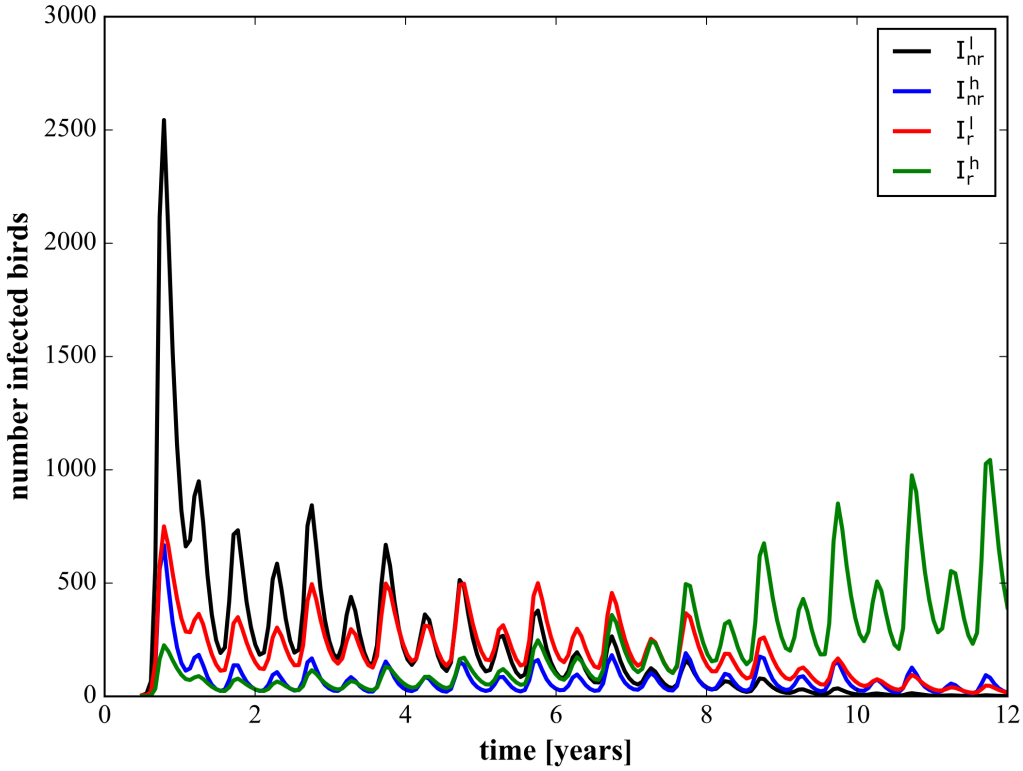



**Figure S3. Stratified prevalence of *M.gallisepticum*.** Timeseries showing infection prevalence in each of the four host classes: $I^l_{nr}$ (black), $I^h_{nr}$(blue), $I^l_r$ (red) and $I^h_r$ (green). Initially, prevalence is highest in $I^l_{nr}$ (susceptible hosts infected with low virulence *Mg*) because high rates of mortality in susceptible birds (initially the most common host genotype) put high virulence strains at a disadvantage. However, as the prevalence of the resistant host phenotype increases, prevalence of the high virulence bacterial strain increases, as carrying the resistance trait reduces the overall fitness costs imposed by the bacteria and thus allows for the evolution towards higher levels of virulence. Parameter values: $\rho_1 = 1, \rho_2 = 1, \rho_3 = 0.1, \lambda_3 = 2$; other parameter values as in Table 1 (main text).

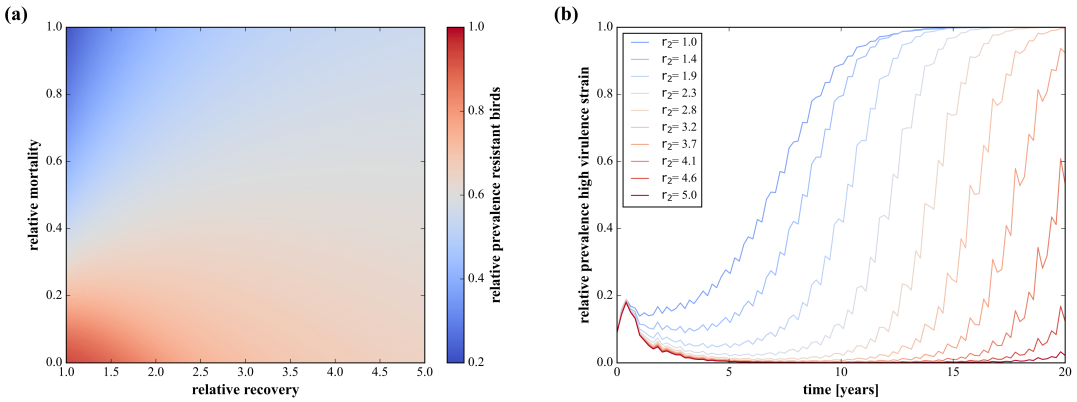
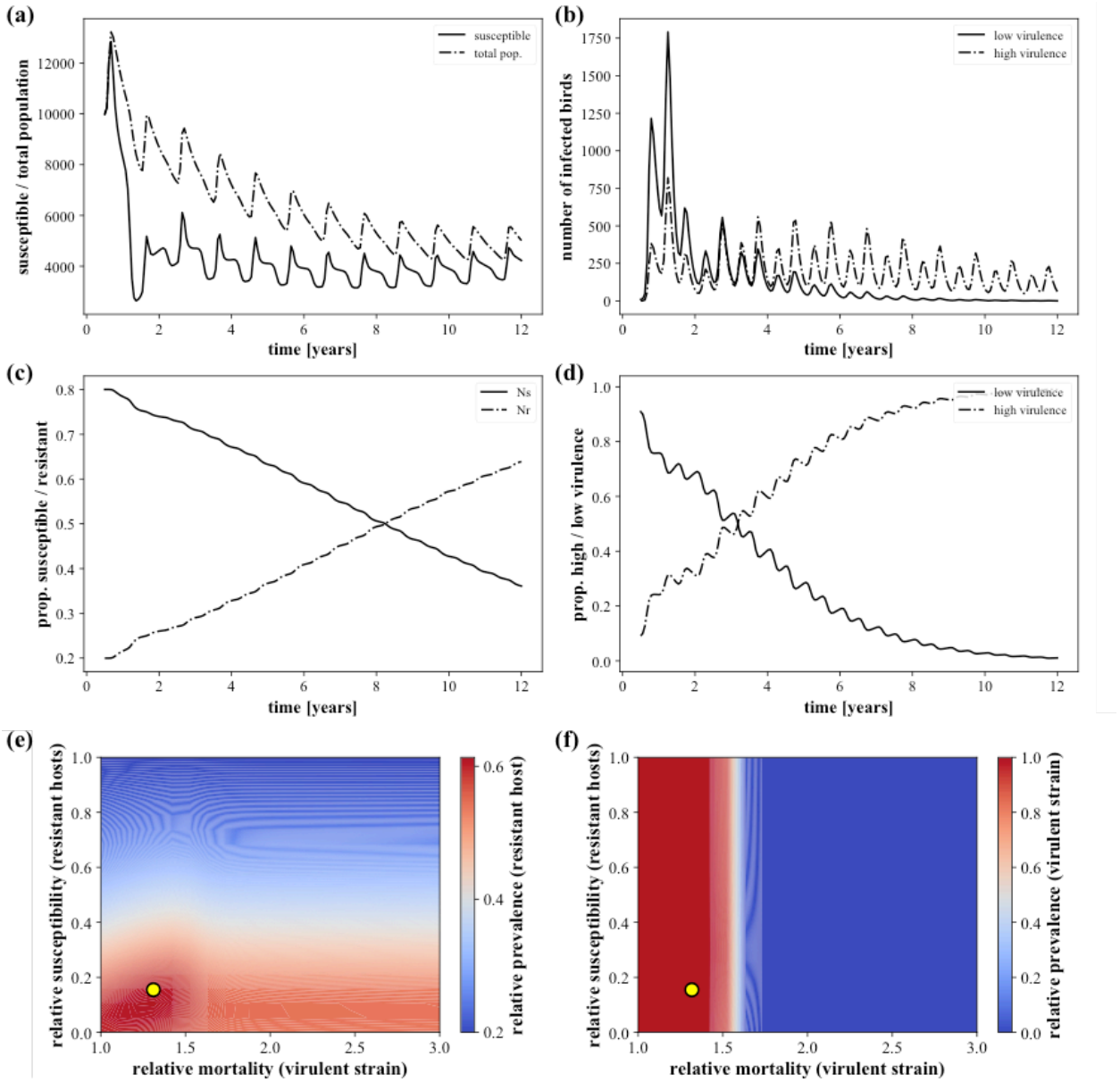



**Figure S4. The effects of the recovery rate on the selection of host resistance and bacterial virulence.** (a) Relative prevalence of the resistance host phenotype 10 years post emergence. Increasing the rate at which birds recover from infection leads to slower selection of resistance due to the overall reduction in infection prevalence and hence selection pressure. (b) Timeseries showing the relative frequency of the high virulence *Mg* strain. With an increase in the recovery rate and therefore decrease in selection pressure, there is a reduction in the rate of host phenotypic change, leading to decrease in the rate at which the more virulent bacterial strain becomes dominant. Parameter values: (a) $\lambda_3 = 2$; (b) $\lambda_3 = 2, \rho_1 = 1, \rho_3 = 0.1$ ; other parameter values as in Table 1 (main text).




1963

1964

1965 **Figure S5: The effect of relative susceptibility on the selection of host**
1966 **resistance and bacterial virulence. (**a) Following disease emergence, there
1967 is a significant decrease in the number of susceptible (solid line) and total
1968 number of hosts (dashed line) in the population over time. (b) Number of
1969 individuals infected with the low virulence (solid line) and high virulence strain
1970 (dashed line) over time showing initial dominance of the low virulent strain,
1971 which eventually gets outcompeted. (c) Change in the proportion of the host
1972 population with a non-resistant or resistant phenotype ($N_{nr}$ and $Nr$,
1973 respectively). (d) Proportion of host population infected with low or high
1974 virulence strains over time showing an initial expansion of the low virulence
1975 strain until the number of resistant hosts has reached a critical threshold that
1976 tips the balance in favour of the more virulent strain. Parameter values:
1977 $\rho_1 = 0.18, \lambda_3 = 1.3$; other parameter values as in Table 1 (main text). (e)
1978 Relative prevalence of the resistance host phenotype 10 years post
1979 emergence. Decreasing susceptibility results in slower selection of host

resistance due to the decrease in overall infection prevalence and selection pressure. (f) Relative prevalence of the virulent bacterial strain 10 years post emergence. High levels of disease-induced mortality rates will select for decreased virulence regardless of susceptibility to infection. Yellow circles indicate parameter values used in (a)-(d).

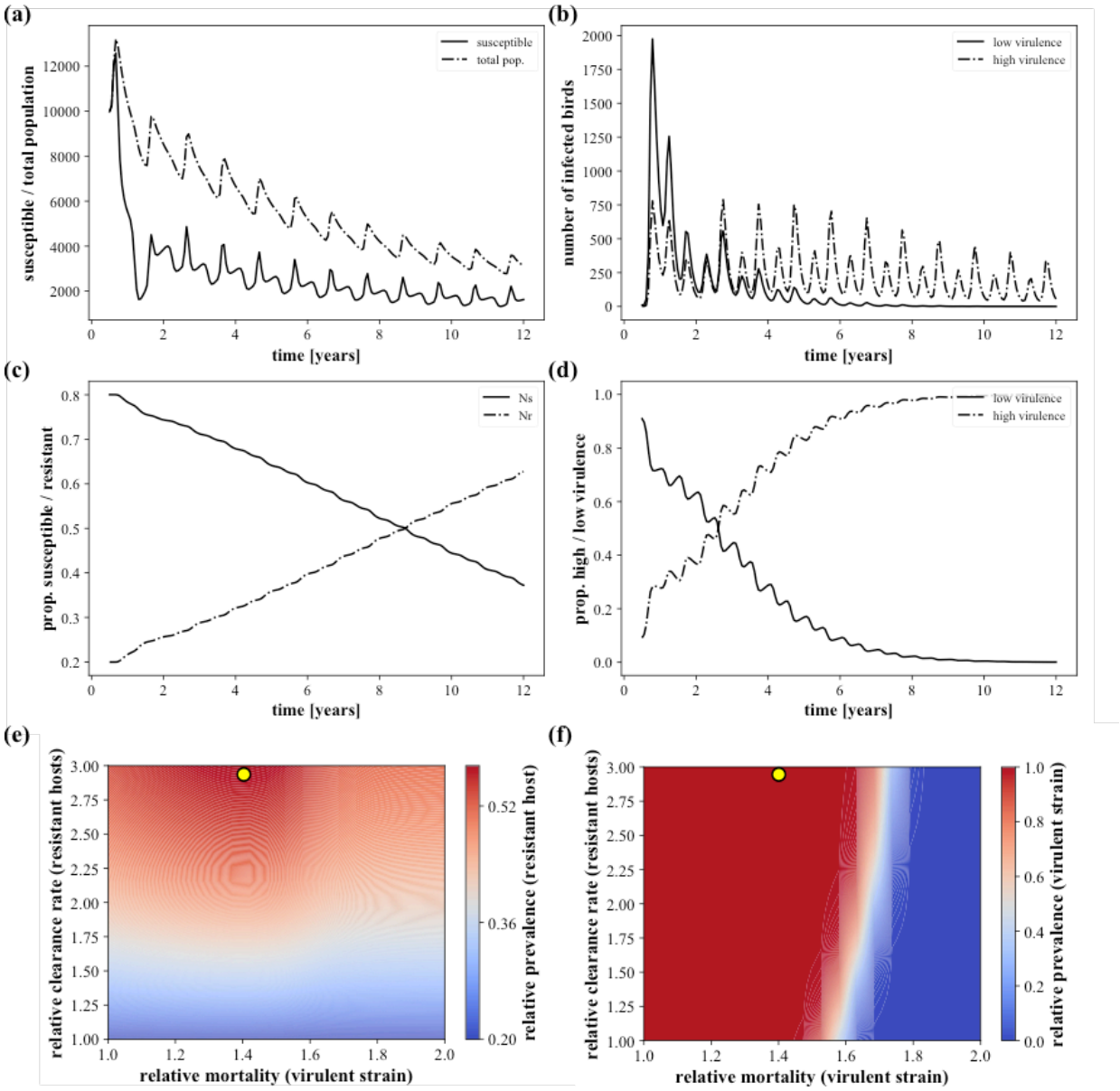



**Figure S6. The effect of parasite clearance on the selection of host resistance and bacterial virulence. (**a) Following disease emergence, there is a significant decrease in the number of susceptible (solid line) and total number of hosts (dashed line) in the population over time. (b) Number of individuals infected with the low virulence (solid line) and high virulence strain (dashed line) over time showing initial dominance of the low virulent strain, which eventually gets outcompeted. (c) Change in the proportion of the host population with a non-resistant or resistant phenotype ($N_{nr}$ and $Nr$, respectively). (d) Proportion of host population infected with low or high

virulence strains over time showing an initial expansion of the low virulence strain until the number of resistant hosts has reached a critical threshold that tips the balance in favour of the more virulent strain. Parameter values: $\rho_2 = 3, \lambda_3 = 1.4$; other parameter values as in Table 1 (main text). (e) Relative prevalence of the resistance host phenotype 10 years post emergence. Increasing the rate at which hosts recover from infection leads to slower selection of resistance due to the overall reduction in infection prevalence and hence selection pressure. (f) Relative prevalence of the virulent bacterial strain 10 years post emergence. Increasing the rate at which hosts recover from infection still results in the more virulent pathogen dominating transmission after ten years, as infection associated death is not so high that the pathogen's transmission is impeded. Yellow circles indicate parameter values used in (a)-(d).

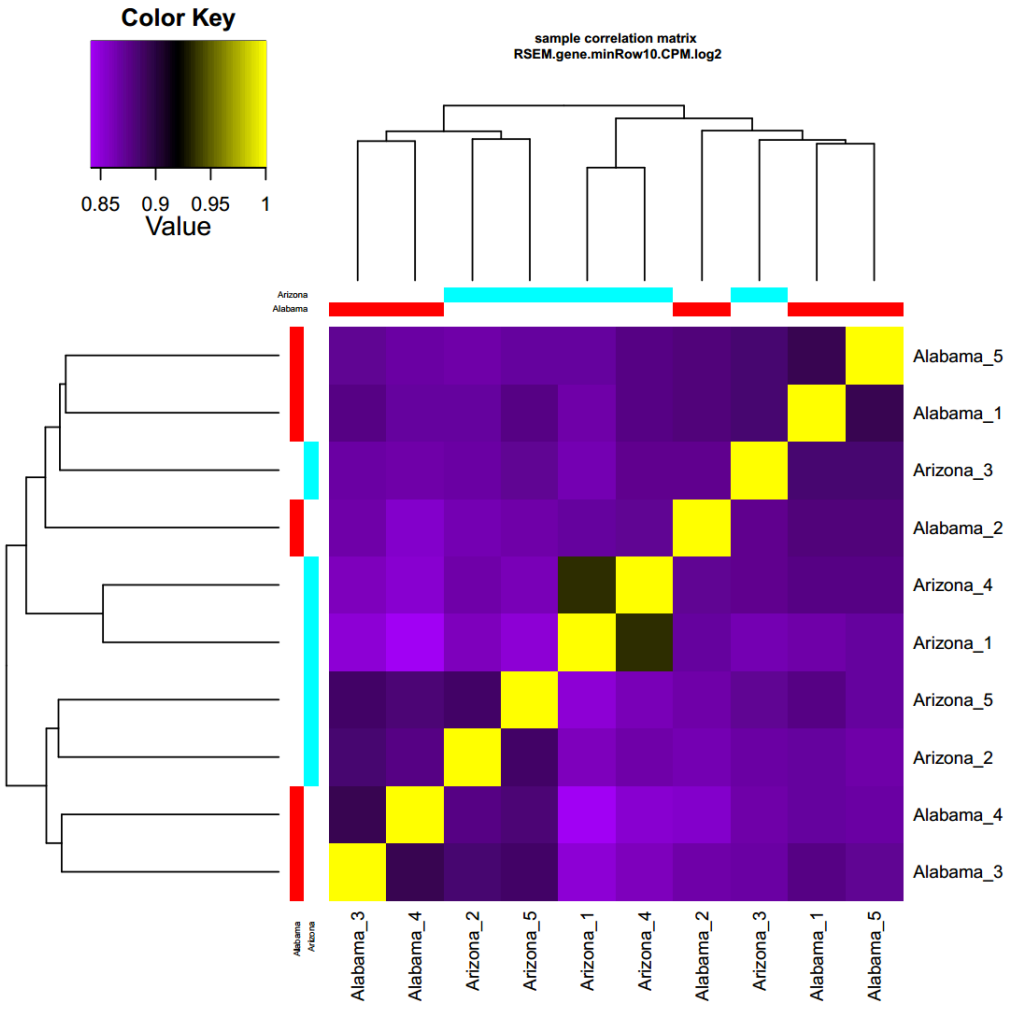



**Figure S7: RSEM correlation matrix of sample similarity.** Stark differences are shown in the transcriptional profiles between Alabama and Arizona individuals. Similarity decreases by colour scale, with yellow representing 100% similarity

## Table S1: Differentially expressed transcripts between Alabama and Arizona finches.

| Vertebrate Homologue | LFC | GO Biological Process | Species |
|---|---|---|---|
| Insulin receptor substrate 4 | -14.83 | insulin receptor signaling pathway | *Zonotrichia albicollis* |
| WD repeat-containing protein 81 isoform X1 | -14.23 | aggrephagy | *Serinus canaria* |
| Probable ATP-dependent RNA helicase DDX17 isoform X2 | -13.43 | androgen receptor signaling pathway | *Falco peregrinus* |
| Basic leucine zipper and W2 domain-containing protein 1 | -12.11 | regulation of transcription, DNA-templated | *Serinus canaria/Taeniopygia guttata/Zonotrichia albicollis* |
| Putative spermidine/spermine N1-acetyl transferase 1 variant 4 | -11.78 | angiogenesis | *Taeniopygia guttata* |
| Cyclin-dependent kinase 19-like, partial | -11.69 | positive regulation of inflammatory response | *Cariama cristata* |
| DNA polymerase kappa isoform X1 | -11.62 | cellular response to DNA damage stimulus | *Serinus canaria* |
| DNA-binding protein RFX7 isoform X2 | -11.61 | regulation of transcription from RNA polymerase II promoter | *Parus major* |
| Cullin-4B | -11.52 | cell cycle | *Apaloderma vittatum* |
| Muscleblind-like protein 1 isoform X14 | -11.48 | myoblast differentiation | *Serinus canaria* |
| Golgin subfamily B member 1 isoform X1 | -11.41 | ER to Golgi vesicle-mediated transport | *Serinus canaria* |
| WD repeat-containing protein 81 isoform X1 | -11.05 | aggrephagy | *Serinus canaria* |
| Mast cell protease 1A-like | -10.85 | proteolysis | *Serinus canaria* |
| Hypothetical protein AV530_004877 | -10.84 | unknown | |
| Nesprin-3 isoform X4 | -10.67 | cytoskeletal anchoring at nuclear membrane | *Serinus canaria* |
| Sorting nexin-3 isoform X1 | -10.67 | intralumenal vesicle formation | *Ficedula albicollis* |
| Eukaryotic translation initiation factor 5 | -10.63 | activation of GTPase activity | *Serinus canaria* |
| Protein LLP homolog | -10.59 | dendrite extension | *Corvus brachyrhynchos* |
| Muscleblind-like protein 1 | -10.48 | myoblast differentiation | *Leptonychotes weddellii* |
| Nuclear pore complex protein Nup214 isoform X1 | -10.33 | intracellular transport of virus | *Serinus canaria* |
| F-box only protein 30 | -10.22 | post-translational protein modification | *Serinus canaria* |
| DnaJ homolog subfamily A member 2 | -9.95 | positive regulation of cell proliferation | *Zonotrichia albicollis* |
| Akirin-2 isoform X1 | -9.83 | innate immune response | *Falco peregrinus* |
| Tuberin-like | -9.43 | unknown | *Melopsittacus undulatus* |
| Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha isoform | -9.39 | adaptive immune response | *Dasypus novemcinctus* |
| E3 UFM1-protein ligase 1 | -9.19 | negative regulation of apoptotic process | *Corvus cornix cornix* |
| Putative ATP-dependent RNA helicase DDX5, partial | -9.17 | alternative mRNA splicing, via spliceosome | *Charadrius vociferus* |
| Hypothetical protein UY3_18385 | -9.04 | unknown | *Chelonia mydas* |
| Cytosolic acyl coenzyme A thioester hydrolase | -9.04 | acyl-CoA metabolic process | *Fukomys damarensis* |
| Lysine-specific demethylase 3B, partial | -9.02 | histone H3-K9 demethylation | *Serinus canaria* |
| Dipeptidyl peptidase 2 isoform X3 | -8.94 | proteolysis | *Serinus canaria* |
| Protein FAM172A-like, partial | -8.93 | unknown | *Haliaeetus albicilla* |
| ATP-dependent RNA helicase SUPV3L1, mitochondrial | -8.93 | chromatin maintenance | *Aquila chrysaetos canadensis* |
| Transcriptional adapter 1, partial | -8.89 | histone H3 acetylation | *Haliaeetus albicilla* |
| Mas-related G-protein coupled receptor member A1-like | -8.89 | chemosensory behavior | *Zonotrichia albicollis* |
| tRNA selenocysteine 1-associated protein 1-like isoform X4 | -8.81 | selenocysteine incorporation | *Zonotrichia albicollis* |
| Peptidase M20 domain-containing protein 2, partial | -8.77 | unknown | *Geospiza fortis* |
| PHD finger protein 10 isoform X2 | -8.70 | nervous system development | *Pseudopodoces humilis* |
| ATP-dependent RNA helicase DDX42, partial | -8.68 | protein localization | *Phalacrocorax carbo* |
| Glycogenin-1 isoform X2 | -8.64 | unknown | *Serinus canaria* |

| | | | |
|---|---|---|---|
| Protein fantom isoform X6 | -8.56 | brain development | *Serinus canaria* |
| Uncharacterized protein LOC108963215 | -8.56 | unknown | *Serinus canaria* |
| Bcl-2-associated transcription factor 1 isoform X5 | -8.46 | apoptotic process | *Balearica regulorum gibbericeps* |
| Tyrosine-protein kinase Fer-like | -8.38 | actin cytoskeleton reorganization | *Haliaeetus albicilla* |
| Conserved oligomeric Golgi complex subunit 1 | -8.37 | ER to Golgi vesicle-mediated transport | *Taeniopygia guttata* |
| Sodium channel protein type 5 subunit alpha, partial | -8.32 | trial cardiac muscle cell action potential | *Leptosomus discolor* |
| Lysophosphatidylcholine acyltransferase 1 | -8.27 | neutrophil degranulation | *Zonotrichia albicollis* |
| Protein phosphatase 1M, partial | -8.21 | unknown | *Parus major* |
| Apoptotic protease-activating factor 1 isoform X2 | -8.16 | activation of cysteine-type endopeptidase activity involved in apoptotic process | *Sturnus vulgaris* |
| Origin recognition complex subunit 3 isoform X2 | -8.15 | DNA replication | *Serinus canaria* |
| Bcl-2-associated transcription factor 1 isoform X3 | -8.06 | apoptotic process | *Serinus canaria* |
| Protein yippee-like 2 | -8.01 | unknown | *Alligator mississippiensis* |
| Ectonucleotide pyrophosphatase/phosphodiesterase family member 3 | -7.86 | immune response | *Serinus canaria* |
| Fas-activated serine/threonine kinase | -7.47 | apoptotic signaling pathway | *Sturnus vulgaris* |
| ATP-binding cassette sub-family A member 2 isoform X4 | -7.07 | unknown | *Serinus canaria* |
| Tankyrase-2 isoform X1 | -5.57 | multicellular organism growth | *Parus major* |
| SUN domain-containing ossification factor isoform X3 | -3.71 | multicellular organism development | *Serinus canaria* |
| RNA-binding protein 5 | 13.44 | apoptotic process | *Geospiza fortis* |
| BAG family molecular chaperone regulator 5 isoform X1 | 12.69 | Golgi organization | *Zonotrichia albicollis* |
| Katanin p60 ATPase-containing subunit A1 isoform X1 | 12.68 | cell cycle | *Serinus canaria* |
| Hypothetical protein UY3_18385 | 12.35 | unknown | *Chelonia mydas* |
| AFG3-like protein 2 | 11.95 | axonogenesis | *Geospiza fortis* |
| Uncharacterized protein LOC108447830 | 11.77 | unknown | *Corvus brachyrhynchos* |
| Eukaryotic translation initiation factor 5 | 11.29 | activation of GTPase activity | *Serinus canaria* |
| Myotubularin-related protein 8 | 11.22 | negative regulation of autophagy | *Serinus canaria* |
| Receptor tyrosine-protein kinase erbB-3 precursor | 11.06 | cranial nerve development | |
| Nesprin-1 | 10.97 | cytoskeletal anchoring at nuclear membrane | *Serinus canaria* |
| Calcium uptake protein 2, mitochondrial isoform X2 | 10.94 | mitochondrial calcium ion homeostasis | *Zonotrichia albicollis* |
| Eukaryotic translation initiation factor 5 | 10.78 | activation of GTPase activity | *Serinus canaria* |
| Coiled-coil domain-containing protein 117 isoform X2 | 10.71 | unknown | *Sturnus vulgaris* |
| Eukaryotic translation initiation factor 5 | 10.66 | activation of GTPase activity | *Serinus canaria* |
| Phosphatase and actin regulator 4 isoform X5 | 10.59 | actin cytoskeleton organization | *Serinus canaria* |
| AP-1 complex subunit gamma-1, partial | 9.92 | antigen processing and presentation of exogenous peptide antigen via MHC class II | *Anas platyrhynchos* |
| NF-kappa-B-activating protein | 9.61 | T cell differentiation in thymus | *Aquila chrysaetos canadensis* |
| Transmembrane protein C9orf91 homolog | 9.59 | unknown | *Geospiza fortis* |
| Hypothetical protein Y956_04768, partial | 9.58 | unknown | *Nipponia nippon* |
| Centrosomal protein of 290 kDa isoform X7 | 9.52 | cilium assembly | *Serinus canaria* |
| UAP56-interacting factor isoform X2 | 9.48 | mRNA export from nucleus | *Parus major* |
| UAP56-interacting factor-like isoform X1 | 9.22 | mRNA export from nucleus | *Zonotrichia albicollis* |
| NADH dehydrogenase [ubiquinone] flavoprotein 3, mitochondrial | 9.19 | mitochondrial ATP synthesis coupled electron transport | *Serinus canaria* |
| Probable ATP-dependent RNA helicase DHX40 | 9.13 | mRNA splicing, via spliceosome | *Serinus canaria* |
| Phosphatidylinositol transfer protein beta isoform isoform X1 | 9.12 | lipid metabolic process | *Zonotrichia albicollis* |
| Serine/threonine-protein kinase PAK 3-like | 9.09 | axonogenesis | *Serinus canaria* |

| | | | |
|---|---|---|---|
| Endoplasmic reticulum lectin 1 | 9.08 | ERAD pathway | *Serinus canaria* |
| Protein phosphatase Slingshot homolog 1-like, partial | 9.07 | actin cytoskeleton organization | *Gavia stellata* |
| Serine/threonine-protein kinase PAK 3-like | 8.87 | axonogenesis | *Ficedula albicollis* |
| Required for meiotic nuclear division protein 1 homolog isoform X4 | 8.74 | unknown | *Serinus canaria* |
| Protein lin-54 homolog isoform X1 | 8.72 | cell cycle | *Serinus canaria* |
| Inositol polyphosphate 5-phosphatase OCRL-1 isoform X3 | 8.67 | cilium assembly | *Serinus canaria* |
| Hypothetical protein N323_08112, partial | 8.63 | unknown | *Cathartes aura* |
| Endoribonuclease Dicer | 8.57 | apoptotic DNA fragmentation | *Geospiza fortis* |
| Nuclear envelope pore membrane protein POM 121 | 8.36 | intracellular transport of virus | *Serinus canaria* |
| Hypothetical protein N301_07267 | 8.35 | unknown | *Charadrius vociferus* |
| T-lymphoma invasion and metastasis-inducing protein 2 isoform X4 | 8.34 | G-protein coupled receptor signaling pathway | *Taeniopygia guttata* |
| Golgin subfamily A member 4 isoform X6 | 8.32 | Golgi to plasma membrane protein transport | *Serinus canaria* |
| Dynamin-1-like protein isoform X4 | 8.09 | cellular response to oxygen-glucose deprivation | *Serinus canaria* |
| Heparanase isoform X2 | 7.75 | angiogenesis involved in wound healing | *Serinus canaria* |
| Ganglioside-induced differentiation-associated protein 2 isoform X1 | 7.46 | response to retinoic acid | *Zonotrichia albicollis* |
| Hypothetical protein N334_14123, partial | 6.96 | unknown | *Pelecanus crispus* |
| CAMP-dependent protein kinase inhibitor alpha | 6.89 | negative regulation of cAMP-dependent protein kinase activity | *Acanthisitta chloris* |
| Phosphatidate cytidylyltransferase 2 | 6.27 | CDP-diacylglycerol biosynthetic process | *Zonotrichia albicollis* |
| Apoptosis inhibitor 5 isoform | 6.03 | apoptotic process | *Pseudopodoces humilis* |
| Autophagy-related protein 9A | 4.53 | autophagosome assembly | *Serinus canaria* |

Table shows transcripts with a significance value (FDR) <0.01, p-value <0.01 and a log fold change >4 (N=103). The functional annotation of each predicted differentially expressed protein-coding sequence was performed with a BLAST search against the nonredundant (NR) database on NCBI using Blast2GO with an *E*-value threshold of 1e-3.

# Chapter 4: Thesis Discussion

Through theoretically modeling the dynamical interplay between host resistance and pathogen virulence, we have revealed specific host traits that likely led to rapid evolution of both antagonists in just a decade. This work suggests that host resistance evolved through an imperfect strategy, and likely through a specific type of quantitative immunity that reduces host mortality through immune action. Indeed, through previous studies examining splenic gene expression profiles, we have seen changes in the regulation of genes involved with immune action accompanying the evolution of resistance (Bonneaud *et al.*, 2011, 2012b). Subsequently, we tested the efficacy of one immune mechanism at protecting hosts from morbidity and mortality. We found no evidence to suggest that circulating antibodies were associated with an increased likelihood of either pathogen clearance, or recovery from symptoms. This finding is similar to previous findings in chickens, where a link between increased IgY and increased pathogen control was never made (Noormohammadi *et al.*, 2002).

In chapter one, we showed that neither a reduction in host susceptibility to *M. gallisepticum* infections, nor an increase in parasite clearance rates, is compatible with patterns observed in previous studies (Bonneaud *et al.*, 2011; Hawley *et al.*, 2013). In fact, model simulations suggest that the impact of each on infection prevalence would reduce disease-induced selection pressure to the extent that we would no longer be able to observe shifts in resistant and virulent host and pathogen phenotypes. Instead, our results

suggest that the rapid, disease-induced selection of host resistance traits based on reducing infection-associated mortality, for instance through a lowering of pathogen load, was the key driver for the subsequent increase in bacterial virulence.

Comparing transcriptional responses of coevolved versus naïve house finches to infection provided novel insights into the nature of effective immune mechanisms in this system. Interestingly, finches from Arizona up-regulated many genes involved in inflammation, where as those from Alabama showed minimal evidence of an increased inflammatory response. Taken together with the fact that *M.gallisepticum* is known to potentiate inflammatory responses in chickens (Ganapathy & Bradbury, 2003), this is indicative that one mechanism for effective protection from *M.gallisepticum* infection may be to avoid its immune-manipulation capabilities. This may be effective through limiting the ability of the pathogen to create more microlesions, therefore infiltrating epithelial and endothelial tissues. Although this warrants more investigation, this could provide precursory evidence through which finches can limit mortality.

Based on evidence presented here regarding an as yet unknown role of insulin pathways and differential energy usage between populations in response to infection, many unexplored avenues remain. Arizona finches exhibited up-regulation of a gene associated with stress, in line with similar previous findings (Bonneaud *et al.*, 2011). Insulin is directly opposed by the action of cortisol, and therefore infection can sometimes lead to

hyperglycemia through hyperinsulinemia or in extreme cases, insulin resistance (McGuinness, 2005). Future comparison with a non-inoculated individuals will help determine whether Alabama birds in fact down-regulate genes involved with insulin pathways relative to Arizona birds, in which case, this may purely represent differential energy usage between populations. Conversely, our results may demonstrate an interaction between stress and the action of the immune system in unexposed individuals if these genes are up regulated in Arizona birds only; in which case, this may indicate that coevolved populations have evolved a mechanism to avoid this damaging effect.

In light of knowledge gained here regarding the evolution of host immune responses, it would now be useful to understand how corresponding pathogen traits that confer increased virulence have co-evolved during the same time frame. Further study into how these findings relate to host and pathogen fitness will also allow us to investigate potential patterns of local adaptations in this system. Moreover, as we were able to identify specific genes that are up regulated in response to infection, future studies may specifically test the efficacy of such response pathways in conferring protection, as we have done with IgY.

In conclusion, the results presented in this thesis are relevant for other host-pathogen systems, particularly as a demonstration of the power of host-driven processes influencing the evolutionary trajectory of host resistance and pathogen virulence. We know from previous modeling studies that the specific host trait under selection is hypothesized to have serious implications for

whether pathogens evolve increasing or decreasing virulence in response (Gandon & Michalakis, 2000; Restif & Koella, 2003). Furthermore, we know from serial passage experiments that imperfect immunity can result in increases to pathogen virulence (Mackinnon & Read, 2004). Our study emphasizes the need to understand directional evolutionary responses when making decisions about control studies or eradication efforts, because if the strategy is imperfect, increases in pathogen virulence may undermine any effort. In addition, we present a rare example of the use of RNA-seq based transcriptomics to characterize the host transcriptome in response to infection. RNA-seq based transcriptomics has scarcely been applied to wild systems, largely due to the inability to control conditions in wild populations and due to the potential for environmental contamination. To the best of our knowledge, the use of transcriptomics for comparisons of unexposed and coevolved host responses has not yet been done in this system. Finally, and as has been noted elsewhere (Pedersen & Babayan, 2011), immune components are not always protective under all circumstances, which was demonstrated in chapter 3. Therefore, when measuring immune responses, future studies should assess whether they are beneficial in the face of the specific pathogen of interest as this is likely to affect host evolutionary trajectories.

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