

Experimental evidence for distinct costs of pathogenesis and immunity against a natural pathogen in a wild bird

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Abstract

Protective immunity is expected to evolve when the costs of mounting an immune response are less than those of harbouring pathogens. Estimating the costs of immunity vs. pathogenesis in natural systems is challenging, however, because they are typically closely linked. Here we attempt to disentangle the relative cost of each using experimental infections in a natural host–parasite system in which hosts (house finches, *Carpodacus mexicanus*) differ in resistance to a bacterium (*Mycoplasma gallisepticum*, MG), depending on whether they originate from co-evolved or unexposed populations. Experimental infection with a 2007-strain of MG caused finches from co-evolved populations to lose significantly more mass relative to controls, than those from unexposed populations. In addition, infected co-evolved finches that lost the most mass harboured the least amounts of MG, whereas the reverse was true in finches from unexposed populations. Finally, within co-evolved populations, individuals that displayed transcriptional evidence of higher protective immune activity, as indicated by changes in the expression of candidate immune and immune-related genes in a direction consistent with increased resistance to MG, showed greater mass loss and lower MG load. Thus, mass loss appeared to reflect the costs of immunity vs. pathogenesis in co-evolved and unexposed populations, respectively. Our results suggest that resistance can evolve even when the short-term energetic costs of protective immunity exceed those of pathogenesis, providing the longer-term fitness costs of infection are sufficiently high.

Keywords: ecological immunology, host–parasite co-evolution, house finch, *Mycoplasma gallisepticum*, quantitative RT-PCR

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Introduction

Pathogens can have considerable social, conservation and evolutionary impacts because they are ubiquitous with potentially devastating effects on hosts (Morens *et al.* 2004; Kwiatkowski 2005; Barreiro & Quintana-Murci 2010). For example, a number of studies have demonstrated pathogen-driven selection on immune

genes in humans (Ferrer-Admetlla *et al.* 2008; Fumagalli *et al.* 2009), and pathogens have been found to be stronger drivers of local adaptation in humans than climate or diet (Fumagalli *et al.* 2011). Some of the best evidence of the evolutionary significance of pathogens on natural animal populations comes from experiments in the water flea (*Daphnia* spp.), in which pathogens and water flea eggs can be recovered and hatched from pond core samples representing various time points in the past. Infections of daphnia from time (t) with pathogens from time ($t + 1$) are more detrimental than those involving pathogens from ($t - 1$) (Decaestecker *et al.* 2007), and pathogens are known to change the genetic structure of

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water flea populations (Mitchell *et al.* 2004; Duncan & Little 2007). These observations, and others like them, illustrate both the intensity of pathogen-driven selection and the ability of hosts to evolve resistance.

The evolution of defence against pathogens via the activation of an immune response, however, incurs energetic costs. For example, immune challenges in a variety of animals show that immune activation can influence metabolic rates (Martin *et al.* 2003; Eraud *et al.* 2005), nutritional status (Barbosa & Moreno 2004; Povey *et al.* 2009), mass (Ots *et al.* 2001), susceptibility to oxidative stress (Bertrand *et al.* 2006) and investment in a number of life-history traits (e.g. Bonneaud *et al.* 2003; Little & Killick 2007). As a consequence, resistance to disease should evolve only when the costs of pathogenesis outweigh the costs of defence (Behnke *et al.* 1992; Sheldon & Verhulst 1996; Boots & Haraguchi 1999; Siva-Jothy *et al.* 2001; Boots & Bowers 2004). The costs of pathogenesis encompass all costs arising because of the development of disease and resulting consequences, for example, from the pathogen's use of host resources, pathogen-induced host tissue degradation, immune manipulation or increased susceptibility to other pathogens (Hornef *et al.* 2002). Despite significant interest in the costs of disease resistance, the magnitude of immune costs relative to other energetic expenses, including the costs of pathogenesis, remains poorly documented and controversial (Svensson *et al.* 1998; Ots *et al.* 2001; Burness *et al.* 2010). One reason for this is that the costs of immunity are classically assessed following challenges with inert antigens or vaccines to eliminate the confounding effects of the parasite's presence and control for past exposure (Owen *et al.* 2010). As a result, we have limited understanding of the cost of immunity to real pathogens, and we still lack empirical studies comparing the magnitude of energetic costs attributable to immunity vs. those directly or indirectly ensuing from the parasite's presence (Owen *et al.* 2010).

To investigate the costs associated with pathogenesis and resistance to a natural pathogen, we use experimental infections and quantitative amplifications of immune and immune-associated genes in a natural host–parasite system involving the house finch (*Carpodacus mexicanus*) and the bacterium *Mycoplasma gallisepticum* (MG). Two features of this host–parasite system make this study possible. First, although house finches extend across temperate regions of North America (Bock *et al.* 1976; Hill 1993), finches in the eastern part of the range are markedly more resistant to MG than those in the west, a difference that has been shown to have arisen as a result of exposure to MG and subsequent selection for resistance (Bonneaud *et al.* 2011). In the mid-1990s, a devastating outbreak of conjunctivitis caused by MG, a novel parasite to songbirds, resulted in the death of

hundreds of millions of house finches in the eastern half of the species' range (Nolan *et al.* 1998). Experimental infections of wild-caught house finches in Alabama in 1998–1999 revealed MG-mediated mortality rates of 30–90% in aviaries with *ad libitum* food (Roberts *et al.* 2001a; Farmer *et al.* 2002). This percentage is probably exacerbated in the wild because MG causes blindness and immunosuppression; so infected birds are more susceptible to starvation, depredation and secondary infections (Roberts *et al.* 2001b; Hotchkiss *et al.* 2005). Within approximately 3 years of the initial epizootic outbreak, declines of eastern populations as a result of MG infection stabilized (Hochachka & Dhondt 2000); infection experiments on eastern and western birds indicated that resistance against MG evolved in eastern U.S. finches within 12 years of exposure to the disease (Bonneaud *et al.* 2011). On the other hand, house finch populations in western North America, and particularly in southern Arizona, were geographically shielded and experienced no MG infection. Second, MG can manipulate the immune system of susceptible hosts, ensuring that no protective immune response, however ineffective, is activated in susceptible hosts (Naylor *et al.* 1992; Ganapathy & Bradbury 2003; Mohammed *et al.* 2007; Bonneaud *et al.* 2011). *Mycoplasma gallisepticum* infections are indeed associated with the suppression of components of the immune system that are pathogen specific (i.e. T-cell immunity: Ganapathy & Bradbury 2003; Gaunson *et al.* 2000; Mohammed *et al.* 2007; and humoral immunity: Matsuo *et al.* 1978; Naylor *et al.* 1992). To facilitate colonization, however, MG can also have a stimulatory action on other components of host immunity, which therefore take part in mediating MG pathogenicity and are included in the costs of pathogenesis (Razin *et al.* 1998). As such, MG induces a damaging inflammatory response that disrupts the mucosal membrane of the host and allows the systemic dissemination of MG (Gaunson *et al.* 2000, 2006; d'Hauteville *et al.* 2002; Hornef *et al.* 2002).

We conducted an infection experiment on finches originating from eastern U.S. (Alabama) populations, which have coexisted with MG since 1995 and have evolved resistance, and from western U.S. (Arizona) populations with no prior exposure to MG (Bonneaud *et al.* 2011). Importantly, no finches used in this study had previously been exposed to MG, such that none were mounting secondary immune responses to MG. We estimated the costs of immunity and pathogenesis as a change in body condition (i.e. mass corrected for body size) over the course of the experimental infection (14 days). In addition, we examined the transcriptional responses elicited by the infection in the trachea, which is the site of infection, and in the spleen, an important tissue for the orchestration of both innate and acquired

immunity (Mebius & Kraal 2005), to estimate levels of protective immune activity against MG.

First, we verify that the costs of infection in birds from Alabama reflect the costs of protective immune activity, while those in birds from Arizona expose the costs of pathogenesis. This assumption will be upheld if, following experimental infection with MG, birds from Alabama that display low levels of MG show the greatest mass loss, while the reverse is expected in birds from Arizona. Second, we test whether the costs of immunity exceed the costs of pathogenesis, as would be expected given the evolution of resistance in the eastern US finches. We predict that experimentally infected birds from Arizona lose more mass relative to uninfected controls from the same population, than infected birds from Alabama do relative to their controls. Finally, we verify that transcriptional evidence of protective immune activity is associated with reduced MG load across individuals and test whether increased protective immune activity is associated with greater mass loss in finches from Alabama. If true, then this would confirm that protective immune activity is the likely generator of any associations between infection and mass loss in Alabama finches.

Material and methods

Capture, housing and experimental infection

We captured male house finches in suburban habitats of Arizona ($N = 37$) and Alabama ($N = 67$) over several days (3–30), at several sites (distant by 1–100 km) and during the dispersive winter period (Hill 1993), to maximize the chances of capturing individuals from multiple populations within each state. Birds were brought back to indoor housing facilities on the Auburn University campus (Alabama), where they were maintained in identical conditions on *ad libitum* food and water. Following quarantine, prior exposure to MG was investigated using serum plate agglutination assay and amplification of MG DNA from choanal and conjunctival swabs (Luttrell *et al.* 1998; Roberts *et al.* 2001a). Serum agglutination assay has been found to reveal past exposure of house finches to MG at least a year after experimental infection and recovery in the laboratory (G.E. Hill, unpublished data). Overall, 12 birds from the Alabama population were removed from the experiment following evidence of previous exposure to MG; no birds used in this study were found to be currently or previously infected with MG. All birds were housed in *ad libitum* conditions for at least 3 months before experimentation, thus reducing the possibility that responses to infection were affected by short-term differences in condition (see SOM for further

discussion). Twenty-two birds from each state were then randomly selected to be included in this study and subsequently assigned at random to MG-inoculated experimental groups or sham-inoculated controls (Arizona: $N_{\text{controls}} = 11$, $N_{\text{infected}} = 11$; Alabama: $N_{\text{controls}} = 10$, $N_{\text{infected}} = 12$). These individuals were included in a previous microarray-based study examining transcriptional differences between pools of finches from Arizona and from Alabama (Bonneaud *et al.* 2011), allowing us to generate predictions in this study regarding associations between individual level gene expression patterns and levels of resistance to MG.

Control birds were sham-inoculated by dropping 10 μl of sterile SP4 media into each eye; birds in experimental groups were inoculated by dropping 10 μl of a stock culture containing approximately 1×10^4 to 1×10^6 colour-changing units/ml of an MG field isolate collected in Auburn, AL, January 2007 (BUA #243) (Whitcomb 1983). Because MG is transmissible through direct contact between infected finches or contact with an infected feeder (Dhondt *et al.* 2007), control and infected birds were maintained under identical conditions, but in separate rooms of the aviary. Infection (or lack thereof) was confirmed and subsequently monitored through serum plate agglutination assay and amplification of MG DNA from choanal and conjunctival swabs 3, 8 and 14 days postinfection. Fourteen days post-treatment, birds were euthanized under licence, and the spleen, trachea and conjunctivae immediately removed and stored in RNA later (Ambion) at -80°C . All birds were weighed (± 0.01 g) at the start and at the end of the experiment using a top-pan balance.

Quantitative RT-PCRs

We isolated total genomic DNA belonging to both MG and house finches in the conjunctiva of infected house finches using the Qiagen AllPrep DNA/RNA Mini Kit. We then quantified MG load in the conjunctiva using a TaqMan qRT-PCR amplification of the MG single-copy gene *mgc2* (Grodio *et al.* 2008). We amplified the house finch *rag1* gene (also single copied), to control for variation in amounts of starting material (Grodio *et al.* 2008). Reactions were run on an ABI Prism 7500 (Applied Biosystems). We made a standard curve for both genes using a final reaction concentration of 25, 12.5, 6.25, 2.5, 0.25 and 0.025 $\mu\text{g}/1$ of genomic DNA to estimate the relative amount of MG between individuals (Bonneaud *et al.* 2011). *Mycoplasma gallisepticum* load was then estimated by dividing the number of *mgc2* genes by one half the number of *rag1* genes, thereby approximating the ratio of MG cells (haploid) to host cells (diploid).

We extracted total RNA from approximately 17 mg of sonicated spleen tissue using Qiagen RNeasy

miniprep spin columns and followed by DNase digestion of genomic DNA according to the manufacturers' protocol (Turbo DNase, Ambion). We extracted total RNA from approximately 10 mg of homogenized tracheal tissue using Qiagen RNeasy fibrous tissue miniprep spin columns, including on-column digestion of genomic DNA according to the manufacturers' protocols (Qiagen, Valencia, CA, USA). We determined the quantity of purified total RNA using a Nanodrop spectrophotometer and determined RNA integrity on an Agilent 2100 Bioanalyzer (Agilent Technologies). Gene expression changes in the spleens and trachea of all birds were determined using a multiplex quantitative RT-PCR approach (Balenger *et al.* 2012; Bonneaud *et al.* 2011). Briefly, we quantified transcriptional changes at 16 genes using multiplex quantitative real-time amplifications: *immunoglobulin J* (GenBank Accession nos: GW346136), *parathymosin* (DR782728), *MHC class II-associated invariant chain Ii* (DR782801), *immunoglobulin superfamily member 4A isoform a* (GW346137), *TCR beta chain* (DR782813), *heat shock protein 90* (DR782718), *NADH dehydrogenase subunit 4* (DR782776), *thioredoxin* (GW346164), *prosaposin* (GW346155), *eukaryotic translation initiation factor eIF4E* (DR782722), *nucleic acid binding protein RY-1 variant 3* (GW346152), *MAK-like kinase* (GW346139), *RhoA GTPase* (GW346157), *ubiquitin C* (DR782830), *lymphocyte cytosolic protein* (DR782758) and *SEC61 gamma subunit* (GW346159). Genes were selected on the basis that they were significantly differentially expressed in response to experimental infection with MG in a previous microarray experiment (Bonneaud *et al.* 2011). The fluorescently dyed PCR products generated were subsequently examined by capillary electrophoresis (Beckman Coulter CEQ8000) for fragment size determination. Raw expression data for each gene were first normalized against an RNA transcript (KAN^R) provided with the GenomeLab GeXP Start Kit (Beckman Coulter) that was spiked into each reaction to control for technical variation and then against the expression value of a house finch housekeeping gene (*Actin related protein 2/3*) included in each reaction (Balenger *et al.* 2012). The housekeeping gene was selected on the basis that it should be ubiquitously expressed in all eukaryotic cells and was previously found not to be differentially expressed significantly between MG-infected and control house finches (Bonneaud *et al.* 2011). Expression of each of the 16 genes of interest was then quantified based on a standard curve (Balenger *et al.* 2012).

Statistical analyses

All analyses were conducted in Genstat, Release 14 (VSN International, Rothamsted Experimental Station, Harpenden, UK). First, the relationship between MG

load and mass loss in each of the two populations was analysed using a general linear model (GLM), in which the amount of mass lost between the day of infection and 14 days thereafter was fitted as the response term to a Gaussian error structure, and the interaction between population and MG load was fitted as the interaction of interest. MG load was transformed to account for nonlinearity of ratio data, by adding one and then taking the natural logarithm. Tarsus length and mass at day 0 were fitted to account for differences in initial body condition that might influence susceptibility to infection (Klasing 1998). Second, we investigated the relative costs of protective immunity vs. pathogenesis by comparing the mass loss of experimentally infected birds relative to controls from the two populations using a two-way analysis of variance. These analyses allow us to test the underlying assumption that mass loss reflects the costs of protective immunity and pathogenesis in eastern vs. western birds, respectively, and the hypothesis that the costs of the latter exceed that of the former.

Finally, in two subsequent analyses, we verified that protective immune activation is responsible for the differences observed previously by investigating the relationships between protective immune activity and each of MG load and mass loss. In a previous microarray-based experiment, we showed that the 16 genes investigated here were significantly differentially expressed between infected Arizona and infected Alabama birds, with 14 of the 16 genes being more highly expressed in infected Alabama compared with infected Arizona birds, the exceptions being *hsp90* and *eukaryotic translation initiation factor (eIF4E)*, which were more highly expressed in infected Arizona finches (Bonneaud *et al.* 2011). Given that Alabama birds have evolved resistance to MG, while Arizona finches were never exposed to MG and hence remain susceptible (Bonneaud *et al.* 2011), our hypotheses will be verified if postinfection transcriptional patterns, in which expression is up-regulated at those 14 genes and down-regulated at *hsp90* and *eIF4E*, are associated with increased resistance to MG (i.e. lower MG load and greater mass loss). We therefore tested whether variation in transcript levels in a direction consistent with increased resistance to MG (i.e. increased expression at the 14 genes and lowered expression at *hsp90* and *eIF4E* post-infection) was associated with differences in MG load and mass change over the course of the experiment.

To test this, we first standardized normally distributed gene expression levels following (Gelman & Hill 2007, p. 54). Second, we then regressed either MG load or mass loss against the standardized expression level of each gene (in both the spleen and the trachea). Finally, we used the resulting gradients of the regression slopes in

one-sample two-tailed *t*-tests to investigate whether the average of the estimates was significantly different from zero (see SOM for further details). The *t*-tests were conducted separately for analysis of gene expression associations with MG load and mass loss, as well as separately within each for effects in the spleen and the trachea. Data from the two populations were combined in the analyses of MG load because the effects of gene expression on MG load would be expected to be complementary in the two populations, that is, up-regulation (or down-regulation) of genes conferring resistance is true independently of population (Bonneaud *et al.* 2011). By contrast, data from the two populations were analysed in separate *t*-tests in the case of mass loss, because relationships between mass loss and gene expressions in the two populations would be expected to be antagonistic, that is, mass loss in Alabama reflects protective immunity and in Arizona reflects pathogenesis (see SOM for further details).

Results

Mass loss, MG load and treatment

Overall, experimentally infected birds harboured an average MG load (MG: house finch cell ratio) in their conjunctivae of 2.6: 1 after 14 days (± 1.0 SD). However, MG load was 48% greater after 14 days in birds from Arizona than those from Alabama, confirming that, on average, birds from the former population were more susceptible to MG infection than birds from the latter population (student's *t*-test; $T_{21} = 3.12$, $P = 0.005$). In addition, although house finches averaged 18.8 g (± 2.0 g SD) before experimental inoculation with MG, they lost an average of 9% of their mass (mean = 1.8 g \pm 1.4 g S. D.) over the 14-day experiment. Importantly, the relationship between MG load and mass loss differed significantly between the populations (GLM, popula-

tion \times MG load; $F_{1,18} = 10.14$, $P = 0.005$; Fig. 1A, $R^2 = 19\%$). This interaction was generated because birds from Alabama had reduced MG load with increasing mass loss [effect (± 1 SE) = 2.24 ± 1.01 ; GLM, $T_{17} = 2.21$, $P = 0.041$], while the reverse was true among birds from Arizona [effect (± 1 SE) = -3.31 ± 1.39 ; GLM, $T_{17} = -2.32$, $P = 0.033$]. Interestingly, birds from Alabama lost more mass than those from Arizona (GLM population main effect; $F_{1,18} = 2.89$, $P = 0.010$), suggesting that the short-term energetic costs of immunity exceeded those of pathogenesis.

That the costs of immunity exceeded the costs of pathogenesis were confirmed by the comparisons of mass losses of infected vs. control birds in each of the populations. When considered together, sham- and MG-infected finches lost an average of 1.16 g (± 1.25 SD) or 6.0% ($\pm 6.0\%$ SD) of their original mass over the course of the experiment, with infected birds losing ~4-fold more mass than controls (infected vs. controls: -1.75 ± 1.36 vs. -0.43 ± 0.69 ; *t*-test, $T_{33} = 3.87$, $P < 0.001$). However, the mass lost by infected birds differed between birds from the two populations, generating a significant population by treatment interaction (GLM, population \times treatment: $F_{1,40} = 4.57$, $P = 0.039$; $R^2 = 6.2\%$; Fig. 1B). While infected birds from Alabama lost 10 times more mass than controls ($T_{13} = 3.67$, $P = 0.003$), those from Arizona only lost two times more mass than controls (GLM, $T_{20} = 1.90$, $P = 0.072$), resulting in infected birds from Alabama losing three times more mass relative to controls than those from Arizona.

Transcriptional response to MG infection, MG load and mass loss

Patterns of gene expression suggestive of increased resistance to MG were significantly and negatively associated with MG load across birds from both

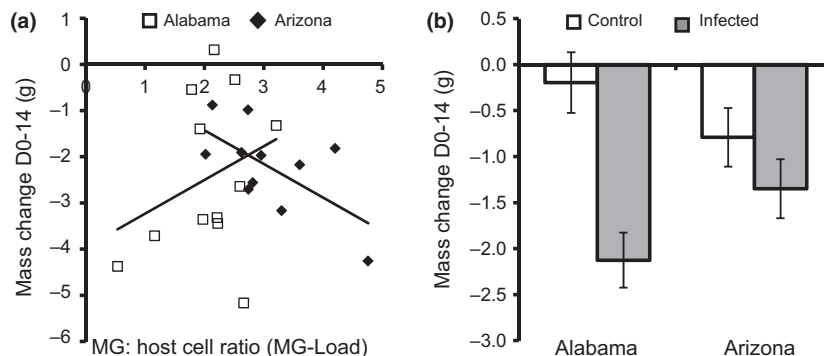


Fig. 1 Mass loss, *Mycoplasma gallisepticum* (MG) load (ratio of MG: host cells in conjunctivae) and treatment. (A) Association between MG load after 14 days and mass change (g) between days 0 and 14 in birds from Alabama (open squares) and Arizona (filled diamonds). Figure shows predicted values (back-transformed) generated from a general linear model and best-fit regression line. (B) Effects of MG- vs. sham inoculations on mass change (g) between days 0 and 14 in birds from both populations. Figure shows means \pm 1 SE.

populations (one-sample two-tailed *t*-test; spleen: $T_{15} = -5.54$, $P < 0.001$; trachea: $T_{15} = -2.25$, $P = 0.040$; Fig. 2A). In other words, individuals that had the highest levels of protective immune activity (i.e. the most extreme expression changes in a direction suggestive of increased resistance to MG) harboured the lowest levels of MG. In addition, we found a significant association between mass change and expression patterns consistent with increased resistance to MG, but as expected, this association was opposite between populations (paired *t*-tests, spleen: $T_{15} = 3.80$, $P = 0.002$; trachea: $T_{15} = 4.56$, $P < 0.001$; Fig. 2B).

Discussion

Previous microarray-based investigations of the transcriptional response of house finches from Alabama and Arizona to an experimental infection with MG revealed that, while pooled Arizona finches primarily down-regulated immune and immune-related genes postinfection, pooled infected Alabama finches resisted MG-associated immunosuppression and mounted a specific cell-mediated immune response (Bonneaud *et al.* 2011; Bonneaud *et al.* 2012). Here we show that differences in gene expression changes postinfection were associated with population differences in mass loss over the course of the infection, with infected Alabama finches losing more mass than infected Arizona finches. We argue that this difference in mass loss reflects differences in the short-term energetic costs resulting from the activation of a protective immune response vs. those

arising from MG pathogenesis, in Alabama and Arizona birds, respectively. In support of this interpretation, we show that, in Alabama, the infected birds that lost the most mass also harboured the least amounts of MG in their conjunctivae, while the reverse was true in Arizona. In addition, protective immune activity, as evidenced by splenic and tracheal gene expression patterns, negatively correlated with MG load. Finally, infected Alabama finches displaying highest levels of protective immune activity exhibited the greatest mass loss over the course of the experiment.

To understand these patterns more fully, it is important to outline the mechanisms that underlie MG pathogenicity and host immune defence against MG, most of which has been identified in poultry. In chickens, MG causes a respiratory disease, which, in the acute phase of the infection, is accompanied by pathological lesions in tissues such as the trachea, air sacs, conjunctivae and the lungs (Javed *et al.* 2005). These lesions result from the induction of an inflammatory response, and the activation and recruitment of a large number of inflammatory cells, such as heterophils, macrophages and lymphocytes to the mucosal tissues (Dykstra *et al.* 1985; Gaunson *et al.* 2000; Javed *et al.* 2005). The ability of MG to elicit such a response (for e.g. by activating B-lymphocyte proliferation in a nonspecific polyclonal way; Razin *et al.* 1998; Simecka *et al.* 1993) is thought to play a major role in MG pathogenicity by facilitating host invasion and immune avoidance (Hornef *et al.* 2002; Javed *et al.* 2005). On the other hand, clearance and recovery from MG infections in previously vacci-

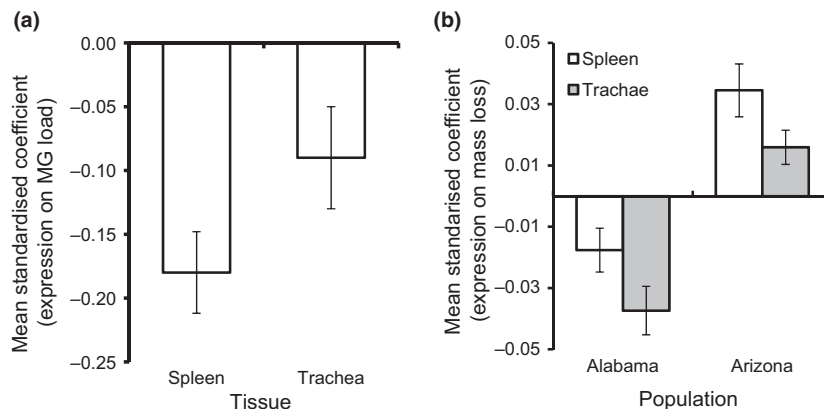


Fig. 2 *Mycoplasma gallisepticum* (MG) load and mass change as a function of protective immune activity, determined by gene expression levels in the spleen and trachea of infected house finches. (A) MG load as a function of protective immune activity in infected finches (Alabama and Arizona shown together); we show the mean (± 1 SE) of the estimates of the regressions of MG load on each of the 16 genes included in the multiplex qRT-PCR. Protective immune activity [i.e. the higher (or lower) expression levels of genes in the same direction as the expression patterns associated with greater resistance to MG (Bonneaud *et al.* 2011)] was negatively associated with MG load in both spleen and tracheal tissues. (B) Mass change as a function of protective immune activity in infected finches (Alabama and Arizona shown separately); we show the mean (± 1 SE) of the estimates of the regressions of mass loss on each of the 16 genes amplified. Protective immune activity was associated with a greater mass loss in Alabama.

nated chickens have been shown to require a relatively rapid cell-mediated immune response (Mukherjee *et al.* 1990; Gaunson *et al.* 2000, 2006), as well as systemic and local antibody responses (Avakian & Ley 1993; Reddy *et al.* 1998). Resistance, however, is determined primarily by the local action of immunoglobulin in the tracheal mucosa at the time of initial colonization of MG (Papazisi *et al.* 2002). Given that inflammation (Bonneaud *et al.* 2003; Owen-Ashley & Wingfield 2006), lesions as well as cell-mediated and humoral immunity (Martin *et al.* 2003; Eraud *et al.* 2005) have been shown to be both energetically and evolutionary costly, observations from poultry suggest that both the presence of MG and the activation of protective immunity against MG should incur a cost. Evidence from studies of clinical symptoms and transcriptional changes postinfection with MG suggests that similar immune processes are occurring in house finches infected with MG (Farmer *et al.* 2002; Bonneaud *et al.* 2011).

Our ability to isolate the costs of pathogenesis from the costs of protective immunity in this wild host-parasite system stems primarily from the fact that eastern and western house finch populations differ in their history of exposure to MG. Indeed, Arizona house finches, which have historically never experienced an MG outbreak, were shown to down-regulate immune or immune-related genes (with the exception of genes restricting immunity) postinfection, while Alabama finches, which had evolved resistance to MG within 12 years of exposure, were able to resist immunosuppression and mounted an immune response that was protective, as evidenced by reduced MG load postinfection (Bonneaud *et al.* 2011). As a result, evidence for a cost of infection in Arizona finches can only be attributed to the cost of pathogenesis, that is, the costs resulting from the presence of MG and a combination of the use of host resources, manipulation of host immunity (e.g. stimulation of an inflammatory response), malfunctions caused by tissue degradation and/or the energetic requirements of tissue repair because of inappropriate or excessive immune activation. Despite previous evidence of costs associated with inflammation (Bonneaud *et al.* 2003; Owen-Ashley & Wingfield 2006), infected Arizona birds only showed a nonsignificant tendency to lose more mass over the course of the experiment than controls, although they still lost more than double the mass of their controls. Food provided *ad libitum* and a relatively low sample size might have contributed to this lack of statistical significance. Either way, these results suggest that the changes in mass detected in infected Alabama birds cannot be primarily attributed to the costs of pathogenesis, as we would expect such costs to be even lower in infected finches from Alabama (Bonneaud *et al.* 2011).

Significantly greater mass loss in experimentally infected Alabama finches compared with their controls should therefore primarily reflect the costs ensuing from the activation of a protective immune response against MG. Indeed, the transcriptional response to infection with MG revealed that these birds activated cell-mediated immune processes, which were associated with functional benefits in terms of lower MG load (Bonneaud *et al.* 2011; Bonneaud *et al.* 2012). Increased energetic expenditure as a result of immune activation is supported by the fact that infected Alabama finches have been previously shown to express higher levels of genes encoding for proteins belonging to the mitochondrial electron transport chain (NADH dehydrogenase subunit 4, cytochrome b and cytochrome c oxidase subunits I-IV) than infected finches from Arizona (Bonneaud *et al.* 2011). However, a lack of compensation of such immune costs, despite access to *ad lib* food supplies, suggests that depletion of host resource for defence may have also partly resulted from an adaptive, illness-induced anorexia. Illness-induced anorexia is an active host defence mechanism that is activated by the immune system (Murray & Murray 1979; Exton 1997), and the costs incurred as a result of anorexia are therefore included in the costs of protective immunity. Such anorexia is thought to be beneficial to infected hosts, in part because it may restrict the availability of key nutrients important to pathogens (Hart 1988; Kyriazakis *et al.* 1998) and because it increases immunocompetence (for e.g. by decreasing resource allocation to other activities such as digestion (Adamo 2010)). Thus, it may be a particularly efficient resistance strategy against obligatory pathogens such as MG, which rely entirely on host resources to survive (Razin *et al.* 1998).

Although our evidence suggests that mass loss as a result of MG infection resulted from the costs of protective immunity in birds from Alabama and from the costs of pathogenesis in those from Alabama, counter to our expectation, the costs of the former appear to outweigh those of the latter. Nevertheless, that this conclusion is based on two independent comparisons would suggest that it is robust. First, infected birds from Alabama lost more mass than those from Arizona over the 14-day experiment. Second, infected birds from Alabama lost substantially more mass relative to their controls than did those from Arizona. For resistance to have evolved in eastern finches, despite greater energetic costs of protective immunity, requires that these costs be offset by higher long-term cost of pathogenesis. In other words, if the selection pressure is strong enough, then protective immunity might evolve irrespective of the energetic costs associated with doing so. The MG epizootic was estimated to have directly or indirectly led to the death of hundreds of millions of

eastern house finches (Nolan *et al.* 1998). Individuals carrying a mutation conferring resistance to MG would thus have been at a distinct selective advantage, even if they suffered substantial energetic costs associated with mounting a protective immune response against MG. This hypothesis leads to the prediction that protective immune activation in eastern house finches should be independent of condition; in support, we failed to find any evidence to suggest that MG load after 14 days was influenced by initial body condition (mass correcting for tarsus; GLM, $F_{1,10} = 0.09$, $P = 0.77$).

In conclusion, our results suggest that in this host–parasite system, the cues mediating the activation of a protective immune response are independent of current condition or consequential short-term costs, and are probably to be genetically determined. However, the patterns that we observe here clearly need further testing before generalizations can be attempted. We thus hope that this study will encourage further attempts to quantify the relative costs of protective immunity and pathogenesis in natural host–parasite systems. In particular, there is a need to test these findings not only in captive conditions, but also in the field where food is more limiting and other threats to survival such as predation and secondary infections are present.

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The authors study host-parasite interactions to understand the evolutionary consequences of disease outbreak (C.B.), and how they relate to the evolution of ornamental traits in vertebrates (S.L.B. and G.E.H.). AFR studies cooperation and conflict in wild populations.

Data accessibility

The sequence data are deposited on NCBI GenBank with Accession nos: DR782718, nDR782722, DR782728, DR782758, DR782776, DR782801, DR782813, DR782830, GU937791, GW346136, GW346137, GW346139, GW346152, GW346155, GW346157, GW346159 and GW346164. The qRT-PCR, MG load and mass data are available as Supporting information file (SOM2).

Supporting information

Additional Supporting Information may be found in the online version of this article.

Appendix S1. Supplementary material.

Data S1. Raw data.

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