

## Innate immunity and the evolution of resistance to an emerging infectious disease in a wild bird

CAMILLE BONNEAUD,<sup>\*†</sup> SUSAN L. BALENGER,<sup>‡¶</sup> JIANGWEN ZHANG,<sup>§</sup> SCOTT V. EDWARDS<sup>\*</sup> and GEOFFREY E. HILL<sup>‡</sup>

<sup>\*</sup>Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, MA 02138, USA, <sup>†</sup>Station d'Ecologie Expérimentale du CNRS (USR2936), 09200 Moulis, France, <sup>‡</sup>Department of Biological Sciences, Auburn University, Auburn, AL 36849, USA, <sup>§</sup>Faculty of Arts and Sciences, Information Technology Research Computing, Harvard University, Cambridge, MA 02138, USA

### Abstract

Innate immunity is expected to play a primary role in conferring resistance to novel infectious diseases, but few studies have attempted to examine its role in the evolution of resistance to emerging pathogens in wild vertebrate populations. Here, we used experimental infections and cDNA microarrays to examine whether changes in the innate and/or acquired immune responses likely accompanied the emergence of resistance in house finches (*Carpodacus mexicanus*) in the eastern United States subject to a recent outbreak of conjunctivitis-causing bacterium (*Mycoplasma gallisepticum*—MG). Three days following experimental infection with MG, we observed differences in the splenic transcriptional responses between house finches from eastern U.S. populations, with a 12-year history of MG exposure, versus western U.S. populations, with no history of exposure to MG. In particular, western birds down-regulated gene expression, while eastern finches showed no expression change relative to controls. Studies involving poultry have shown that MG can manipulate host immunity, and our observations suggest that pathogen manipulation occurred only in finches from the western populations, outside the range of MG. Fourteen days after infection, eastern finches, but not western finches, up-regulated genes associated with acquired immunity (cell-mediated immunity) relative to controls. These observations suggest population differences in the temporal course of the response to infection with MG and imply that innate immune processes were targets of selection in response to MG in the eastern U.S. population.

**Keywords:** cell-mediated immunity, immunosuppression, microevolution, time-series microarray

Received 20 August 2011; revision received 10 February 2012; accepted 14 February 2012

### Introduction

Novel pathogens are powerful selective agents in humans (Diamond 1997) and other animals (Haldane 1949; Grenfell & Dobson 1995). Studies simultaneously monitoring the emergence of an infectious disease and the associated molecular changes in wild vertebrate host populations are rare, leading to a poor understanding of how hosts evolve immunity to novel pathogens.

Correspondence: Camille Bonneaud, Fax: +33 5 61 96 08 51; E-mail: bonneaud@dr14.cnrs.fr

<sup>¶</sup>Present address: Division of Genetics and Physiology, Department of Biology, University of Turku, Turku FIN-20014, Finland.

One exception involves the study of rapid evolution of disease resistance in Australian populations of the European rabbit (*Oryctolagus cuniculus*) infected with myxomatosis (Kerr & Best 1998). The myxoma virus was released in 1950 and spread rapidly throughout the susceptible Australian rabbit population. Within a few years, however, resistance emerged, apparently mediated through the rabbits' escape from pathogen-induced immunosuppression, which facilitated the development of an enhanced innate, and then a specific cell-mediated immune response (Best & Kerr 2000). Although we know that wild vertebrate host populations can evolve resistance to novel pathogens rapidly (Marshall & Fenner 1958; Bonneaud *et al.* 2011), whether or not such

resistance is typically mediated through initial changes to innate immunity, as the study of rabbits would suggest, is unclear.

Despite the potential for innate immunity to play a key role in the response to novel pathogens, the vast majority of studies in ecological immunology in vertebrates have focused on the acquired immune system (Acevedo-Whitehouse & Cunningham 2006; van der Most *et al.* 2011). The most likely reason for this trend is that most host-pathogen systems studied are assumed to be co-evolving. Unlike innate immunity, responses of acquired immunity are usually pathogen-specific and, therefore, represent more targeted and effective defensive responses, particularly against known pathogens (Janeway 2005). For example, of particular interest in host-parasite co-evolution has been the role of the polymorphic *Mhc* genes in detecting foreign antigens and triggering pathogen-specific T-lymphocyte cytotoxicity and humoral immune responses (Soley & Roberts 1994; Sommer 2005; Piertney & Oliver 2006; Spurgin & Richardson 2010). However, during the early stages of a novel infectious outbreak, pathogen-specific recognition alleles may either be absent or at such low frequencies in host populations that such populations are ill-equipped to deal with novel pathogens. Under such conditions, the spread of adaptive alleles may be slow and stochastic (Wright 1955; Hedrick 2002). By contrast, innate immunity comprises immediate, nonspecific immune processes that are triggered when pattern recognition receptors detect a limited repertoire of conserved but common microbial patterns (e.g. LPS) (Janeway 1989). As a result, innate immunity provides the first line of protection against most pathogenic attacks and can stem infections while pathogen-specific processes are being activated (Janeway 2005). As such, we might expect innate immunity to play a particularly important role during outbreaks of novel infectious diseases. This is particularly true when novel disease outbreaks involve pathogens that are able to manipulate and avoid immune detection, because detection by the acquired immune system (e.g. by *Mhc* molecules) requires their prior recognition and presentation by cells of the innate immune system (e.g. macrophages, dendritic cells) (Iwasaki & Medzhitov 2010). Thus, given the primary role of innate immunity in nonspecifically fighting infections and in regulating acquired immune responses, it is likely that the innate immune processes are paramount in driving resistance to novel pathogens, particularly when those pathogens are able to avoid immune detection.

Here, we make use of the natural epizootic of conjunctivitis caused by the bacterium *Mycoplasma gallisepticum* (MG) in a North American songbird, the house finch (*Carpodacus mexicanus*) (Fischer *et al.* 1997; Dhondt

*et al.* 1998), to investigate the contribution of innate and acquired immunity to the evolution of resistance to a novel pathogen. Mycoplasmosis was first reported in house finches in Maryland in 1994 (Ley *et al.* 1996), the result of a host shift from domestic chickens and turkeys (Delaney *et al.* 2012). Following the initial outbreak, MG spreads rapidly through eastern populations of house finches in North America. The severity of MG as a house finch pathogen early in the epizootic was confirmed by high mortality rates of naturally and experimentally infected finches maintained in captivity (Luttrell *et al.* 1998; Roberts *et al.* 2001a; Farmer *et al.* 2002). In the wild, over two hundred million birds were estimated to have died between 1994 and 1998 (Nolan *et al.* 1998), causing a significant decline in the abundance of house finches over the entire eastern portion of their range (Hochachka & Dhondt 2000). The prevalence of MG in house finches subsequently declined (Hartup *et al.* 2001; Roberts *et al.* 2001b), and evidence now suggests that MG has reached endemic levels in eastern North America, presumably at least in part due to the spread of host resistance within 12 years of exposure to MG (Bonneaud *et al.* 2011).

*Mycoplasma* bacteria are known for effectively evading and manipulating host immune defences (for a review see Razin *et al.* 1998). For example, MG maintains a high diversity of cell surface molecules (Chamblaud *et al.* 1999), including surface lipoproteins, and can vary its antigenic composition at the cell surface in response to environmental cues (Baseggio *et al.* 1996; Markham *et al.* 1998). Such antigenic variation allows mycoplasmas to be resistant to phagocytosis in susceptible hosts (Marshall *et al.* 1995). Immuno-modulatory effects include the ability to induce an inflammatory response at the site of infection (Ganapathy & Bradbury 2003; Gaunson *et al.* 2006), causing host lesions (Ley 2008), as well as the ability to suppress other components of host immunity (Javed *et al.* 2007). For example, simultaneous inoculation of poultry with MG and *Haemophilus gallinarum* (Matsuo *et al.* 1978) or avian pneumovirus (Naylor *et al.* 1992) has been found to lower the humoral antibody response to both *H. gallinarum* and pneumovirus in chickens and turkeys, respectively. Finally, MG infection is associated with suppressed T-cell activity 2 weeks after infection (Gaunson *et al.* 2000; Ganapathy & Bradbury 2003).

To examine the contributions of innate and acquired immunity to the evolution of resistance to MG in house finches, we conducted an infection experiment and examined transcriptional responses elicited in the spleen, an important tissue for the organization of both innate and acquired immunity (Meibius & Kraal 2005). Infections with pathogens are known to induce transcriptional responses in hosts (Jenner & Young 2005), and such responses can differ between individuals

displaying varying levels of resistance to infection (Marquis *et al.* 2008). Investigating differences in gene expression profiles between resistant and susceptible hosts in response to experimental infection might, therefore, offer new insights into the genetic basis underlying immunity (Sarson *et al.* 2008; van der Sar *et al.* 2009). In our study, finches originated from either eastern U.S. (Alabama) populations, which have coexisted with MG since the mid-1990s and show evidence of having evolved resistance, or western U.S. (Arizona) populations with no prior exposure to MG (Bonneaud *et al.* 2011). Gene expression changes between infected and control finches were measured 3 and 14 days after experimental infection. Critically, none of the birds used had been previously infected with MG, and so none of the changes in gene expression post-infection could be explained by secondary immune responses against MG. Although immune processes 3 and 14 days postinfection will generally reflect innate and acquired activity, respectively (Lai *et al.* 1987; Hickman-Davis *et al.* 1998; Gaunson *et al.* 2000; Farmer *et al.* 2002), the genes that underpin these processes may both be expressed sharply after, and continue throughout, infection (Raifa & Buchmann 2008; Sarson *et al.* 2008; Caipang *et al.* 2009; van der Sar *et al.* 2009). Thus, investigating the role of innate and acquired immunity in the evolution of resistance to MG, using patterns of gene expression profiles in transcriptional responses to MG infection, will require testing predictions regarding temporal versus geographical differences.

We made two broad predictions regarding the role of innate and acquired immune responses in the evolution of resistance to MG in eastern house finches. First, we predicted that if an evolutionary response to MG in the eastern populations involved innate immunity, then: (i) eastern and western populations would differ in the transcriptional changes observed between control and MG-infected finches 3 days postinfection; (ii) gene expression differences on day 3 would involve significant gene down-regulation in western but not eastern finches; and (iii) genes associated with acquired immunity would be up-regulated in both populations on day 14 only. These predictions arise because transcriptional differences between populations in the early stages of experimental infection would suggest that early acting innate immune processes differ between populations, and down-regulation is expected in Arizona because of the immuno-modulatory effects of MG infection. However, this scenario would support the hypothesis of selection on innate immunity only if genes known to be associated with acquired immunity were not differentially expressed at an early stage of infection. Second, we predicted that if an evolutionary response to MG in the eastern population primarily involved acquired

immunity, then we would observe population differences in transcriptional changes only 14 days after infection, with an up-regulation of genes associated with acquired immunity in eastern finches.

## Material and methods

### *Experimental infection*

In January and February 2007, we captured male house finches from two geographically distant locations: around Tempe, Arizona in the western U.S., where MG had never occurred in house finches through 2007; and around Auburn and Prattville, Alabama in the eastern U.S., where finches had coexisted with MG for 12 years. MG was never reported in Arizona prior to 2009, despite long-term monitoring (Dhondt *et al.* 2006; Toohey *et al.* 2010), and was confirmed absent from the sampling sites in mid-2011 (G. E. Hill; unpublished data). Sampling was conducted at three different suburban sites in both states: in Arizona, sites were 1–2 km apart and the birds were captured over three days; in Alabama, sites were 10–103 km apart and the birds were captured over 30 days. Following capture, birds were immediately transported by plane from Arizona ( $N = 37$ ) and by car within Alabama ( $N = 64$ ) and established in aviaries at Auburn University, Alabama. Finches were held in cages 0.5 m × 0.5 m with two birds per cage for the duration of the study. Cages were kept indoors, in temperature-controlled rooms with natural light through windows (day-length was unregulated but comparable to the locales from which the birds were captured). Captive finches were fed sunflower seed, brown and white millet, grit, and water *ad libitum*, as well as apple slices and crushed eggshells weekly. The housing conditions, food and day-length regime were identical and represented novel conditions for birds from both populations.

To confirm that the finches had not been infected with MG prior to our study, individuals from Alabama and Arizona were quarantined in separate rooms for the first month. Following quarantine, birds had a blood sample taken via brachial venipuncture (~60 µL of whole blood). Whole blood was tested for MG antibodies using serum plate agglutination assay (SPA), a reliable means of determining prior exposure to MG (Luttrell *et al.* 1996). All birds in the study were further tested for exposure to MG via amplification of MG DNA from choanal and conjunctival swabs (Roberts *et al.* 2001a). Twelve birds from the Alabama population were removed from the experiment when they showed evidence of exposure to MG (eight were symptomatic at capture, one developed symptoms during quarantine, and three were seropositive for MG anti-

bodies). In addition, a further nine from Arizona and 20 from Alabama were used in a different experiment, leaving 28 Arizona birds and 32 Alabama birds in this study.

Birds were randomly selected to be kept either as controls or infected via ocular inoculation with 20 µL of culture containing  $1 \times 10^4$  to  $1 \times 10^6$  colour changing units/mL of an early 2007 Auburn MG isolate. All infected birds were inoculated with precisely the same volume of the same culture. Control birds were sham infected using sterile SP4 medium (Whitcomb 1983). Control ( $N = 11$  birds from Arizona and nine from Alabama) and infected birds were maintained under identical conditions, but in separate rooms of an 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 days postinfection (all treatment groups), as well as 8 and 14 days postinfection for control and infected day 14 treatment groups. Infected birds were euthanized 3 days ( $N = 6$  from Arizona and  $N = 11$  from Alabama) and 14 days ( $N = 11$  from Arizona and  $N = 12$  from Alabama) after treatment. Control birds were euthanized 14 days after sham-inoculation; there was no reason for gene expression differences to occur between 3 and 14 days postinoculation in control birds. The spleens from all birds were removed immediately after euthanization, stored in RNAlater (Ambion) and placed at  $-80^{\circ}\text{C}$ . Sample sizes within groups and the number of groups were kept to a minimum, while still ensuring sufficient statistical power, to adhere to animal ethics stipulations. In addition, the protocol of the experimental infection was specifically designed to allow us to address multiple, orthogonal questions, thereby ensuring that we maximized the use of the data collected (Bonneaud *et al.* 2011; this study).

We acknowledge that a negative consequence of these ethical considerations is the reduced sampling of multiple populations, particularly in the western ranges. Despite the potential problems of between-population comparisons, we believe that the results presented here are robust. First, all birds were acclimatized in the same novel captive environment with *ad libitum* food and water for at least three months prior to the experiment, and comparisons of expression profiles were then made through comparisons of infected versus control birds from each population, rather than simply of infected birds between the two populations. These considerations reduce the risk of obtaining spurious results due for example, to background differences in condition or gene expression. Second, previous comparisons of gene expression patterns between finches from Arizona (2007) and from Alabama early (i.e. in 2000) and later

(i.e. in 2007) in the MG epizootic revealed that as resistance to MG evolved in the eastern populations, expression patterns of Alabama finches became less similar to those patterns found in finches from unexposed Arizona populations (Bonneaud *et al.* 2011). This means that unknown ecological differences between birds from the two populations are unlikely to mediate interpopulation differences in responses to infection with MG. Third, Alabama finches in 2007 harboured lower bacterial loads postinfection and up-regulated immune genes, while finches from Arizona showed evidence of being immunosuppressed (Bonneaud *et al.* 2011). These observations support the interpretation that population differences in responses to MG between Arizona and Alabama finches arose as a consequence of different histories of exposure to the disease.

#### *Sample preparation and microarray hybridization and analysis*

Molecular methods and analyses are detailed in Bonneaud *et al.* (2011). Briefly, we extracted total RNA from approximately 17 mg of spleen tissue using Qiagen RNeasy miniprep spin columns and followed by DNase digestion of genomic DNA according to the manufacturers' protocols. We determined the quantity of purified total RNA using a Nanodrop spectrophotometer and determined RNA integrity on an Agilent 2100 Bioanalyzer. All RNA extracts were stored at  $-80^{\circ}\text{C}$  until further processing.

The samples were hybridized onto a microarray printed with a selection of cDNA clones from two suppression subtractive hybridization libraries (Bonneaud *et al.* 2011). These libraries are enriched in clones differentially expressed between MG-infected and control house finches 2-week postinfection ( $N = 16\,512$  clones) (Wang *et al.* 2006). Obviously, using libraries enriched in cDNA differentially expressed 14 days postinfection precluded us from an exhaustive inventory of the innate immune genes differentially expressed in response to infection between populations. Nevertheless, our microarray is well suited for testing the predictions outlined in the introduction for the following two reasons. (i) MG pathogenesis is largely mediated via the activation of an innate immune response (inflammation) that causes lesions to host tissues (Ley 2008). Consequently, we expect finches to up-regulate innate immune genes 3 days postinfection regardless of whether this reflects MG pathogenesis or increased host resistance via the activation of a protective immune response. By contrast, by 14 days postinfection, finches from Alabama are able to mount a protective immune response to MG, while those from Arizona are immunosuppressed (Bonneaud *et al.* 2011). Hence, by using our

microarray enriched with genes differentially expressed 14 days postinfection, we increased our chances of examining expression changes at genes associated with protective immunity only and eliminated risks that patterns of expression would reflect manipulation of host immunity as a result of MG infection. (ii) If innate or acquired immune processes are to be activated at all, we expect both to be so by 14 days postinfection. In contrast, by 3 days postinfection, acquired immune processes might yet to be activated (Janeway 2005). Hence, a microarray enriched with genes differentially expressed 14 days postinfection will have increased ability to reveal differential gene expression patterns involved with both innate and acquired immune processes, thus allowing us to discriminate between the predictions tested.

Of all the clones present in the libraries, 220 were previously identified as significantly differentially expressed between infected and controls using a macroarray approach (Wang *et al.* 2006). The microarray here consisted of unique amplicons of these 220 clones, as well as amplicons of 694 randomly selected clones from the enriched libraries (Bonneaud *et al.* 2011). Additionally, it contained five house finch housekeeping genes (*Actin-related protein 2/3*, *ATP synthetase*, *ATPase V1 subunit G1*, *Basic transcription factor 3* and *Calmodulin 2*) and 11 *Escherichia coli* housekeeping genes (*arcA*, *aroE*, *dnaE*, *gapA*, *gnd*, *icdA*, *pgm*, *polB*, *putin*, *trpA* and *trpB*; Hommais *et al.* 2005; Noller *et al.* 2003) to facilitate normalization procedures. All clones were printed twice on each grid and each grid was replicated on each half microarray slide, such that all clones were represented four times on a microarray slide.

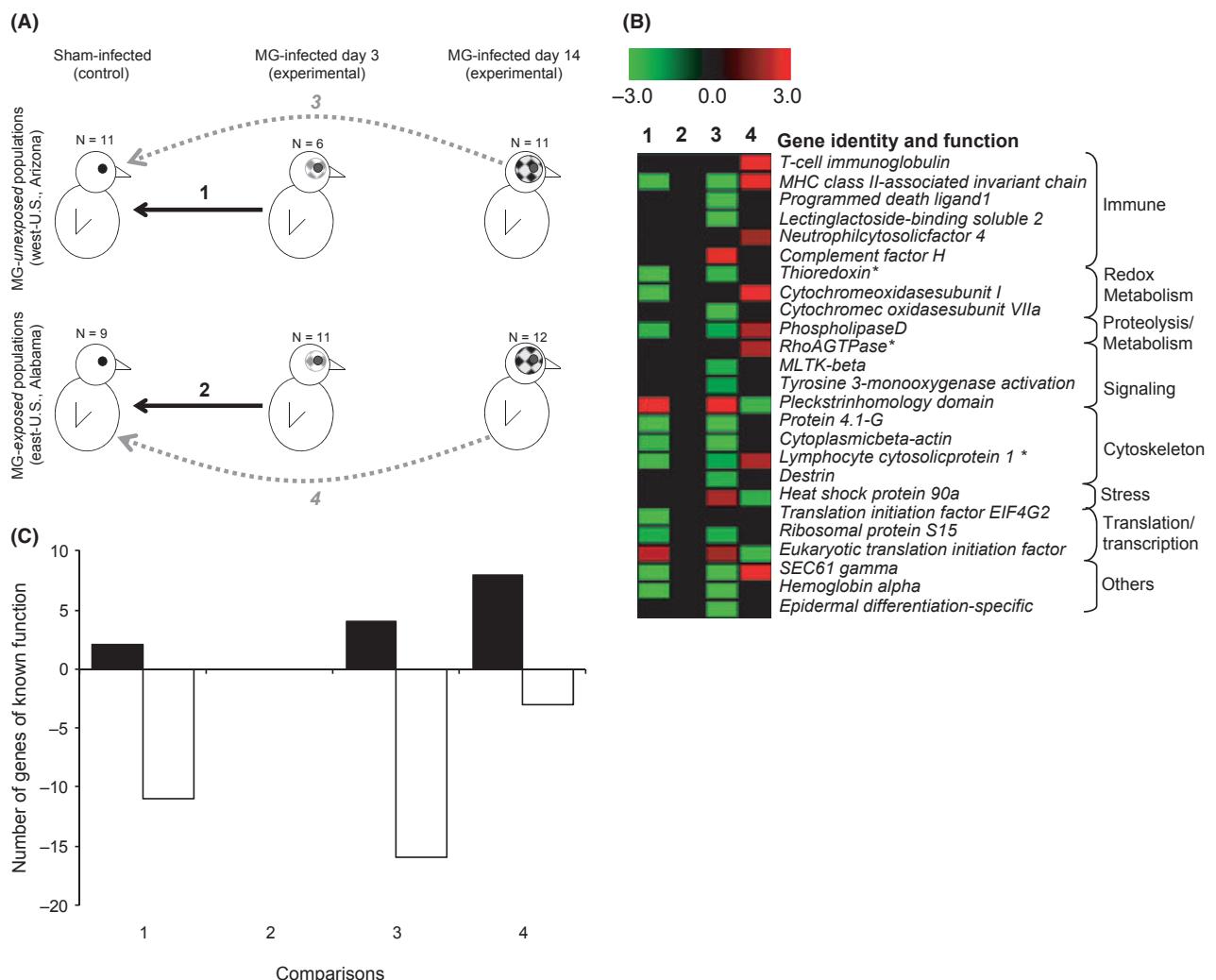
We used a common reference design (Yang & Speed 2002), in which we pooled two to six spleens from birds from the same population in the same treatment to generate enough mRNA for microarray hybridizations and hybridized two pools for each treatment from each population. Whilst pooling precludes investigations of within-pool variation, experiments using appropriate RNA pooling strategies have been shown to improve the accuracy and power of experimental designs in which two arrays are used for each treatment (Peng *et al.* 2003; Kendzierski *et al.* 2005). For example, experiments employing pooling have been found to display consistently smaller within-treatment variability than those with approximately the same number of arrays that did not employ pooling, thereby highlighting meaningful differences between experimental treatments (Peng *et al.* 2003; Kendzierski *et al.* 2005). Each pooled house finch RNA sample was prepared for cDNA microarray hybridization by reverse-transcribing 15 µg of total pooled RNA using amino allyl-dUTP. Pools of cDNA were then purified and labelled using

Cy5 dye and hybridized against a common reference, made by pooling an aliquot of all the individual samples from all treatments and labelled with Cy3.

We used the software package GENEPIX to yield log base-2 measurements for mean fluorescence intensities for each dye channel in each spot on the array and to flag low quality spots. We normalized the log base-2 measurements of mean fluorescence intensities for each dye channel in each remaining spot on the array using R software (<http://www.r-project.org>) and a Matlab interface (MArray), which permits graphical representation of normalized values (Wang *et al.* 2002). The normalization procedure involves adjustments for background dye levels, as well as spatial biases within and among arrays. Detection of within-hybridization (within-slide) problems was facilitated by the two technical replicates of each clone on each array. Detection of within- and between-hybridization (among-slides) problems is also facilitated through the use of external reference *E. coli* RNA and house finch housekeeping genes, both of which generate spots of known intensity values across all slides. The final normalized signal ratios were then fitted to linear model for microarray data (LIMMA) in an R Bioconductor package. LIMMA allows statistical comparisons to be conducted between the two mean expression values from the infected pools with the two from the control pools. It then attributes a significance to each set of comparisons for each clone, with significance attributed when  $P < 0.05$ , but after correcting for false-discovery rates which substantially reduces the probability of reporting Type I errors (Benjamini & Hochberg 1995). All differentially expressed clones were sequenced on an ABI 377 sequencer. Forward and reverse sequences generating a BLAST hit with an *e*-value  $<1 \times 10^{-20}$  and with more than 100 nucleotides were categorized by their vertebrate orthologs, while all other sequenced genes were considered to be unknown. Gene ontology category and function were determined using Harvester (<http://harvester.fzk.de/harvester/>).

### Comparisons

To test our predictions, we made four comparisons of transcriptional responses to MG infection between finches (Fig. 1A). We compared expression differences between infected birds on day 3 postinfection vs. controls in Arizona (1) and Alabama (2), as well as those differences between infected birds on day 14 postinfection vs. controls in Arizona (3) and Alabama (4). Differences in gene expression patterns were analysed using comparisons of observed versus expected frequencies in binomial test and contingency tables (comparing two or more than two independent frequencies, respectively).



**Fig. 1** Comparisons and patterns of splenic gene expression. (A) Schematic of the analytical comparisons made: (1) infected on day 3 postinoculation vs. controls in MG-unexposed Arizona; (2) infected on day 3 postinoculation vs. controls in MG-exposed Alabama; (3) infected on day 14 postinoculation vs. controls in Arizona; (4) infected day 14 postinoculation vs. controls in Alabama. Comparisons (3) and (4) were previously published in Bonneauaud *et al.* (2011). (B) Heat map of gene expression patterns for the 25 genes in comparisons 1–4 above (infected vs. controls). The 25 genes are all those showing differential expression in at least one comparison (1–4) and of known function. Values in red and green indicate significantly higher and lower expression levels, respectively, in comparisons 1–4 above, with bright colours reflecting at least a threefold difference in magnitude and values in black indicating no difference. Gene functions and identities are shown on the right; asterisks indicate genes with an identified auxiliary immune function. The overall down-regulation of gene expression patterns in Arizona birds 14 days postinfection was previously found to be associated with a 50% greater bacterial load compared to infected finches from Alabama (Bonneaud *et al.* 2011). (C) Total number of genes of known function up-regulated (black) and down-regulated (white) in infected vs. control finches in the comparisons 1–4 above.

## Results

We found 105 clones that were significantly differentially expressed in this study, of which 73 were differentially expressed 3 days after infection and 99 were differentially expressed 14 days after infection. Previous sequencing of these clones revealed 25 vertebrate orthologs (Fig. 1B; Table S1, Supporting information; Bonneauaud *et al.* 2011): 13 and 24 that were

differentially expressed 3 and 14 days after infection, respectively. All other clones were unknown. Gene ontology categories and primary functions of the 25 genes included immunity (six genes), redox metabolism (3), metabolism (1), signal transduction (4), stress (1), cytoskeleton (4), transcription/translation (3), transport (2) and cell differentiation (1). Given that all of these genes are differentially expressed as a result of experimental infection, it is likely they all play some role in

the response to infection. Indeed, in addition to the six genes with direct immune function (*T-cell immunoglobulin and mucin domain containing 4*, *MHC class II-associated invariant chain II*, *programmed death ligand 1*, *lectin galactoside-binding soluble 2 protein*, *neutrophil cytosolic factor 4*, *complement factor H*), three of the 'non-immune' genes above have been shown to have auxiliary immune function [*thioredoxin* (Nordberg & Arner 2001), *RhoA GTPase* (Scheele *et al.* 2007) and *lymphocyte cytosolic protein* (Samstag *et al.* 2003)] (Fig. 1B) (see Supporting information). We can rule out the possibility that our results arise because of differences in cDNA quality or abundance between samples because of our extensive use of within- and between-slide controls (see Material and methods).

All predictions that selection has acted on innate immunity only, or on both innate and acquired immunity, were upheld. Three days postinfection, 13 of the 25 genes identified displayed significant differences in expression between infected and control birds from Arizona (comparison 1), but none did between such birds from Alabama (comparison 2) (Fig. 1B,C) (two-sample binomial test = 3.85,  $P < 0.001$ ). In addition, 85% of those 13 genes differentially expressed on day 3 between infected and control birds in Arizona were down-regulated (one-sample binomial test,  $P = 0.02$ ; Fig. 1B,C). Finally, in Arizona, gene expression profiles between experimental and control birds did not differ significantly between days 3 and 14 post-treatment (13 of 25 genes differentially expressed in comparison 1 and 20 of 25 in comparison 3: contingency table,  $\chi^2 = 0.90$ ,  $P = 0.34$ ), although the power of this test was relatively weak (0.45). Moreover, there was no change in the proportion of genes that were down-regulated between the two time points (contingency table,  $\chi^2 = 0.01$ ,  $P = 0.92$ ; power = 0.95). By contrast, in Alabama, a significantly greater number of genes were expressed in infected birds on day 14 than on day 3 (0 genes in comparison 2, 11 in comparison 4: two-sample binomial test = -3.51,  $P < 0.001$ ), and eight of these 14 genes were up-regulated. Importantly, of these eight genes differentially expressed on day 14 in Alabama, one was identified as having a role in innate immunity (*neutrophil cytosolic factor 4*) and two in acquired immunity (*T-cell immunoglobulin and MHC class II-associated invariant chain*), and none was differentially expressed on day 3. This latter result means that population differences in expression patterns 3 days postinfection are unlikely to be attributed to acquired immune processes.

## Discussion

In a previous study, we presented evidence that eastern U.S. populations of house finches evolved resistance to

a devastating outbreak of MG over a 12-year period (Bonneaud *et al.* 2011). Here, we use a microarray analysis and an experimental infection involving finches from MG-exposed eastern U.S. (Alabama) and unexposed western U.S. (Arizona) populations to investigate whether changes to innate and/or acquired immunity accompanied this evolutionary event. Relative to controls, gene expression profiles of birds from Arizona versus Alabama differed both 3 and 14 days following experimental infection, with infected birds from Arizona showing significant down-regulation of gene expression patterns on both days compared to those from Alabama. Moreover, while gene expression profiles were similar on days 3 and 14 in Arizona finches, in Alabama finches, profiles differed significantly between day 3 and 14. This change in gene expression patterns in Alabama finches was a consequence of the up-regulation of acquired immune processes by day 14 but not on day 3. Interpopulation differences between infected and control birds on days 3 and 14 were, therefore, likely due to differences in innate and acquired immune activity. From these observations, we propose that mutations affecting innate immunity only, or both innate and acquired immunity, have accompanied the evolution of resistance to MG in eastern populations of house finches in the 12 years following the onset of the epizootic.

The conclusion that mutations affecting innate immunity played a role in the evolution of resistance to MG hinges on the corroboration of three key predictions: (i) relative to controls, infected birds from Arizona and Alabama displayed distinct transcriptional responses in the early stages of experimental infection; (ii) expression patterns in Alabama were consistent with increased resistance to MG; and (iii) genes associated with acquired immunity were only up-regulated after population differences in transcription were first observed. These observations also allowed us to reject the hypothesis that mutations associated with acquired immunity alone led to the evolution of resistance to MG among eastern U.S. house finches. Nevertheless, mutations associated with acquired immune processes, in addition to those associated with innate immune processes, may have played a role in the evolution of resistance, as evidenced by the transcriptional differences of infected versus control birds between the two populations on day 14 and within Alabama between days 3 and 14.

Evidence from laboratory mice and rats also suggests a role for both innate and acquired immunity in fighting infections with *Mycoplasmas*, but with innate immunity playing a predominant role in fighting initial infections (Hickman-Davis 2002). For example, while acquired immunity appears to be implicated in controlling the spread of *Mycoplasmas pulmonis* within the

body, innate immunity is important for resistance against acute infections (Cartner *et al.* 1998). Natural killer cells and macrophages, which are important actors of innate immunity, have been shown to play key roles in conferring resistance to *M. pulmonis* (Lai *et al.* 1990; Hickman-Davis *et al.* 1997). In addition, phagocytosis, bacterial killing and the release of reactive nitrogen species by macrophages during *M. pulmonis* and *Mycoplasmas pneumoniae* infections seem to be facilitated by collectins, such as surfactant-associated proteins A (Marshall *et al.* 1995; Hickman-Davis *et al.* 1998; Kalina *et al.* 2000), which represent a major group of pattern recognition proteins of the innate immune system (van de Wetering *et al.* 2004). Surfactant-associated proteins A are encoded by polymorphic genes (reviewed in Floros *et al.* 2009; Ledford *et al.* 2010) and both limit inflammatory responses and interact with T-cells, making them particularly interesting candidate genes to examine in the context of the evolution of resistance to MG in eastern U.S. house finches.

Although studies of the response of mammalian hosts to *Mycoplasmas* suggest a role for both innate and acquired immunity in conferring resistance, the evolutionary origins of resistance to MG could be associated with changes in gene(s) implicated in innate immunity only, given that innate immune processes both precede and play a critical role in the activation of acquired processes (Iwasaki & Medzhitov 2010). Under this hypothesis, any population differences in acquired immunity may simply be a consequence of differences in innate immune activity. Hence, although we are not in a position to distinguish whether mutations associated with innate, or with both innate and acquired, immunity have led to the evolution of resistance to MG in eastern house finches, the transcriptional differences we observed on day 14 may result from a single mutation affecting innate immunity and allowing eastern finches to subsequently trigger an acquired immune response. Our results are reminiscent of those obtained from similar experimental infections of wild rabbits with the *myxoma* virus (Best & Kerr 2000). Resistant rabbits had elevated immune responses within 4 days postinfection, in advance of the subsequent increased cell-mediated immune response at least 6 days after infection. The increased resistance of populations of rabbits having experienced the *myxomatosis* outbreak was, therefore, hypothesized to be mediated by enhanced innate immune activity, which subsequently allowed the development of a specific cell-mediated immune response (Best & Kerr 2000). While mutations arising in genes associated with both innate and acquired immunity may have been subject to natural selection, a more parsimonious scenario may be that a change in the frequency of a single mutation affecting innate immune

processes has been primarily responsible for the evolution of resistance to MG. The speed with which resistance evolved in eastern house finches (Bonneaud *et al.* 2011) and the rarity of mutations conferring phenotypic advantages in evolving populations (Blount *et al.* 2008) suggests that selection is unlikely to have simultaneously favoured the spread of two or more distinct pre-existing alleles, but further work is required to test this hypothesis.

All the transcriptional changes that we observed in Alabama finches occurred in response to experimental infection and hence involve genes that may play a role in conferring resistance. However, we identified three genes that are particularly known to have a direct role in immunity in model organisms and humans, and two genes known to play an auxiliary role in immunity, all of which were up-regulated in Alabama finches postinfection. T-cell immunoglobulin and mucin domain containing (TIM) 4 is a trans-membrane receptor at the surface of antigen-presenting cells (e.g. macrophages and dendritic cells), which ligates TIM1 on the cell surface of naive CD4+ T-cells to activate their differentiation into Th2 cells (Liu *et al.* 2007) and which also mediates the clearance of apoptotic (phosphatidylserine-expressing), antigen-specific T-cells after infection to prevent autoimmunity (Albacker *et al.* 2010). MHC class II-associated invariant chain II (CD74) is a chaperone molecule that plays a role during the assembly of MHC class II molecules within and transport out of the endoplasmic reticulum (Bertolino & RabourdinCombe 1996; Stumptner-Cuvelette & Benaroch 2002). *Neutrophil cytosolic factor 4* encodes a cytosolic regulatory component of the superoxide-producing phagocyte NADPH-oxidase and is essential for phagocytosis-induced oxidant production in neutrophils (Matute *et al.* 2009). Finally, the two auxiliary immune genes (*Rho GTPase* and *lymphocyte cytosolic proteins*) have been shown to take part in processes such as Toll-like receptor signalling (Aderem & Ulevitch 2000), formation of reactive oxygen species during oxidative bursts (Kao *et al.* 2008), leucocytes chemotaxis, motility, phagocytosis and cytotoxicity (Khurana & Leibson 2003; Morley *et al.* 2010; Pazdrak *et al.* 2011) and/or T and B-cells development and activation (Wang *et al.* 2010; Todd *et al.* 2011). Hence, 14 days postinfection, Alabama finches up-regulated genes involved in both innate (e.g. phagocytosis by neutrophils) and acquired (e.g. T-cell activity) immune processes.

*Mycoplasma gallisepticum* infection is known to cause the suppression of certain immune components in the initial stages of infection in chickens as evidenced by the significant down-regulation of cytokines (CCL20, IL8 and IL12) as early as 24 h after exposure (Mohammed *et al.* 2007). These effects can last up to 8 days following

infection (Mohammed *et al.* 2007). The expression profiles above suggest that Arizona finches were immune-suppressed throughout the experimental infection, with the majority of genes being down-regulated, including three genes associated with immunity on day 3 and 5 on day 14. Interestingly, the only immune gene that was up-regulated in infected finches from Arizona (complement factor H) has been found to restrict the activation of the complement cascade in humans (de Cordoba & de Jorge 2008) and hence exhibits a direction of expression change consistent with the suppression of immune activity. Conversely, Alabama finches displayed evidence of resistance to immune manipulation as no immune-related genes were down-regulated on day 3 or day 14. Finally, in line with the study of the rabbits/myxomatosis system wherein immunity against myxomatosis was associated with increased cell-mediated (i.e. T helper-cell activity) rather than humoral (i.e. antibody) responses (Best & Kerr 2000), we found that the two immune genes up-regulated on day 14 in Alabama (*T-cell immunoglobulin* and *MHC class II-associated invariant chain*) were associated with cell-mediated immunity. The proteins encoded by these genes are, however, likely to be involved in processes leading to the activation of a humoral immune response (Janeway 2005). Our results suggest that resistance to MG evolved in the eastern U.S. via the ability to mount an innate immune response followed by a cell-mediated immune response against MG.

Resistance can evolve via increased host ability to physiologically limit pathogen invasion upon contact (avoidance), to clear infections (recovery) or to suffer the costs associated with the presence of the pathogen (tolerance) (Boots & Bowers 1999). Whether clearance of infection is mediated by innate or acquired immune processes should depend on characteristics of both the host and the pathogen, such as host lifespan, pathogen transmission rate and pathogenicity, and host recovery rate (Boots & Bowers 2004). In the initial stages of a novel and severe epizootic outbreak, however, innate immune mechanisms conferring increased resistance may be the target of selection, even if natural selection ultimately leads to the evolution of highly specific acquired immune processes. Our results highlight the importance of identifying not only the genetic correlates of adaptation, but also the molecular and cellular processes underlying phenotypic change to better understand how wild populations respond to natural selection (Shapiro *et al.* 2004; Manceau *et al.* 2011). It is noteworthy that the same immune processes appear to be adopted by different species (house finches and rabbits) in response to emerging pathogens (MG and myxoma virus, respectively), perhaps suggesting that the pathways favoured by natural selection may be comparable across host taxa, although further work is

required before coherent generalizations can be made. While previous studies of temporal transcriptional changes have been used to identify the immune processes associated with increased resistance to infectious diseases in both domestic and laboratory animals (Raida & Buchmann 2008; Sarson *et al.* 2008), ours is the first to attempt to do so in a wild population believed to have evolved disease resistance under pathogen-driven natural selection. Further studies are needed using other house finch populations to verify the generality of our results, as well as other emerging host-pathogen systems in an attempt to determine whether selection on innate immunity might be a general prerequisite to the evolution of resistance to novel pathogens.

## Acknowledgements

We thank A. Russell for help with statistical analyses and valuable comments on the manuscript, K. McGraw for logistics in Arizona, J. Johnson for logistics in Alabama, C. Daly for technical advice/help, M. Alcaide for helpful discussions, and the handling Editor, S. Bensch, and four anonymous referees for constructive comments on the manuscript. This work was funded by a Biogrant from the Office for Vice President for Research at Auburn University, by funds from the Center for Environmental and Cellular Signal Transduction (Auburn University), and by NSF Grant 0923088 to GEH and SVE. CB was supported by Harvard University funds (to SVE, CB), a Marie Curie Reintegration Grant (FP7-PEOPLE-IRG-2008, #239257) (CB), CNRS, France (CB); SLB by a grant from the Alabama Experimental Program to Stimulate Competitive Research (EP-SCoR). Protocols were approved by Auburn University Institutional Animal Care & Use Committee (IACUC) permit (#2007-1179) and Auburn University Institutional Biological Use Authorization (BUA) (#243).

## References

- Acevedo-Whitehouse K, Cunningham AA (2006) Is MHC enough for understanding wildlife immunogenetics? *Trends in Ecology & Evolution*, **21**, 433–438.
- Aderem A, Ulevitch RJ (2000) Toll-like receptors in the induction of the innate immune response. *Nature*, **406**, 782–787.
- Albacker LA, Karisola P, Chang Y-J *et al.* (2010) TIM-4, a receptor for phosphatidylserine, controls adaptive immunity by regulating the removal of antigen-specific T cells. *Journal of Immunology*, **185**, 6839–6849.
- Baseggio N, Glew MD, Markham PF, Whithear KG, Browning GF (1996) Size and genomic location of the pMGA multigene family of *Mycoplasma gallisepticum*. *Microbiology*, **142**, 1429–1435.
- Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate – a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society Series B-Methodological*, **57**, 289–300.
- Bertolino P, RabourdinCombe C (1996) The MHC class II-associated invariant chain: a molecule with multiple roles in MHC class II biosynthesis and antigen presentation to CD4(+) T cells. *Critical Reviews in Immunology*, **16**, 359–379.

- Best SM, Kerr PJ (2000) Coevolution of host and virus: the pathogenesis of virulent and attenuated strains of myxoma virus in resistant and susceptible European rabbits. *Virology*, **267**, 36–48.
- Blount ZD, Borland CZ, Lenski RE (2008) Historical contingency and the evolution of a key innovation in an experimental population of *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America*, **105**, 7899–7906.
- Bonneaud C, Balenger S, Russell AF et al. (2011) Rapid evolution of disease resistance is accompanied by functional changes in gene expression in a wild bird. *Proceedings of the National Academy of Sciences of the United States of America*, **108**, 7866–7871.
- Boots M, Bowers RG (1999) Three mechanisms of host resistance to microparasites – avoidance, recovery and tolerance – show different evolutionary dynamics. *Journal of Theoretical Biology*, **201**, 13–23.
- Boots M, Bowers RG (2004) The evolution of resistance through costly acquired immunity. *Proceedings of the Royal Society of London Series B-Biological Sciences*, **271**, 715–723.
- Caipang CMA, Brinchmann MF, Kiron V (2009) Profiling gene expression in the spleen of Atlantic cod, *Gadus morhua* upon vaccination with *Vibrio anguillarum* antigen. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology*, **153**, 261–267.
- Cartner SC, Lindsey JR, Gibbs-Erwin J, Cassell GH, Simecka JW (1998) Roles of innate and adaptive immunity in respiratory mycoplasmosis. *Infection and Immunity*, **66**, 3485–3491.
- Chamblaud I, Wroblewski H, Blanchard A (1999) Interactions between mycoplasma lipoproteins and the host immune system. *Trends in Microbiology*, **7**, 493–499.
- de Cordoba SR, de Jorge EG (2008) Translational mini-review series on complement factor H: genetics and disease associations of human complement factor H. *Clinical and Experimental Immunology*, **151**, 1–13.
- Delaney NF, Balenger S, Bonneaud C et al. (2012) Ultrafast evolution and loss of CRISPRs following host shift in a novel wildlife pathogen, *Mycoplasma gallisepticum*. *Plos Genetics*, **8**, e1002511. doi:10.1371/journal.pgen.
- Dhondt AA, Tessaglia DL, Slothower RL (1998) Epidemic mycoplasmal conjunctivitis in house finches from Eastern North America. *Journal of Wildlife Diseases*, **34**, 265–280.
- Dhondt AA, Badyaev AV, Dobson AP et al. (2006) Dynamics of mycoplasmal conjunctivitis in the native and introduced range of the host. *EcoHealth*, **3**, 95–102.
- Diamond J (1997) *Guns, Germs, and Steel*. W. W. Norton & Company, New York City, New York.
- Edgar R, Domrachev M, Lash AE (2002) Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Research*, **30**, 207–210.
- Farmer KL, Hill GE, Roberts SR (2002) Susceptibility of a naive population of house finches to *Mycoplasma gallisepticum*. *Journal of Wildlife Diseases*, **38**, 282–286.
- Fischer JR, Stallknecht DE, Luttrell MP, Dhondt AA, Converse KA (1997) Mycoplasmal conjunctivitis in wild songbirds: the spread of a new contagious disease in a mobile host population. *Emerging Infectious Diseases*, **3**, 69–72.
- Floros J, Wang GR, Mikerov AN (2009) Genetic complexity of the human innate host defense molecules, surfactant protein A1 (SP-A1) and SP-A2-impact on function. *Critical Reviews in Eukaryotic Gene Expression*, **19**, 125–137.
- Ganapathy K, Bradbury JM (2003) Effects of cyclosporin A on the immune responses and pathogenesis of a virulent strain of *Mycoplasma gallisepticum* in chickens. *Avian Pathology*, **32**, 495–502.
- Gaunson JE, Philip CJ, Whithear KG, Browning GF (2000) Lymphocytic infiltration in the chicken trachea in response to *Mycoplasma gallisepticum* infection. *Microbiology*, **146**, 1223–1229.
- Gaunson JE, Philip CJ, Whithear KG, Browning GF (2006) The cellular immune response in the tracheal mucosa to *Mycoplasma gallisepticum* in vaccinated and unvaccinated chickens in the acute and chronic stages of disease. *Vaccine*, **24**, 2627–2633.
- Grenfell BT, Dobson AP (1995) *Ecology of Infectious Diseases in Natural Populations*. Cambridge University Press, Cambridge.
- Haldane JBS (1949) Disease and evolution. *La Ricerca Scientifica Supplemento A*, **19**, 68–76.
- Hartup BK, Bickal JM, Dhondt AA, Ley DH, Kollias GV (2001) Dynamics of conjunctivitis and *Mycoplasma gallisepticum* infections in house finches. *Auk*, **118**, 327–333.
- Hedrick PW (2002) Pathogen resistance and genetic variation at MHC loci. *Evolution*, **56**, 1902–1908.
- Hickman-Davis JM (2002) Role of innate immunity in respiratory mycoplasma infection. *Frontiers in Bioscience*, **7**, D1347–D1355.
- Hickman-Davis JM, Michalek SM, Gibbs-Erwin J, Lindsey JR (1997) Depletion of alveolar macrophages exacerbates respiratory mycoplasmosis in mycoplasma-resistant C57BL mice but not mycoplasma-susceptible C3H mice. *Infection and Immunity*, **65**, 2278–2282.
- Hickman-Davis JM, Lindsey JR, Zhu S, Matalon S (1998) Surfactant protein A mediates mycoplasmacidal activity of alveolar macrophages. *American Journal of Physiology-Lung Cellular and Molecular Physiology*, **274**, L270–L277.
- Hochachka WM, Dhondt AA (2000) Density-dependent decline of host abundance resulting from a new infectious disease. *Proceedings of the National Academy of Sciences of the United States of America*, **97**, 5303–5306.
- Hommais F, Pereira S, Acquaviva C, Escobar-Paramo P, Denamur E (2005) Single-nucleotide polymorphism phylotyping of *Escherichia coli*. *Applied and Environmental Microbiology*, **71**, 4784–4792.
- Iwasaki A, Medzhitov R (2010) Regulation of adaptive immunity by the innate immune system. *Science*, **327**, 291–295.
- Janeway CA (1989) Approaching the asymptote – evolution and revolution in immunology. *Cold Spring Harbor Symposia on Quantitative Biology*, **54**, 1–13.
- Janeway C (2005) *Immunobiology: The Immune System in Health and Disease*. Garland Science, New York City, New York.
- Javed M, Frasca S, Cecchini K et al. (2007) Chemokine and cytokine gene expression profiles in chickens inoculated with *Mycoplasma gallisepticum* strains R-low or GT5. *Vaccine*, **25**, 8611–8621.
- Jenner RG, Young RA (2005) Insights into host responses against pathogens from transcriptional profiling. *Nature Reviews Microbiology*, **3**, 281–294.
- Kalina M, Blau H, Riklis S, Hoffman V (2000) Modulation of nitric oxide production by lung surfactant in alveolar

- macrophages. *Advances in Experimental Medicine and Biology*, **479**, 37–48.
- Kao YY, Gianni D, Bohl B, Taylor RM, Bokoch GM (2008) Identification of a conserved Rac-binding site on NADPH oxidases supports a direct GTPase regulatory mechanism. *Journal of Biological Chemistry*, **283**, 12736–12746.
- Kendzierski C, Irizarry RA, Chen KS, Haag JD, Gould MN (2005) On the utility of pooling biological samples in microarray experiments. *Proceedings of the National Academy of Sciences of the United States of America*, **102**, 4252–4257.
- Kerr PJ, Best SM (1998) Myxoma virus in rabbits. *Revue Scientifique Et Technique De L'Office Internationale Des Epizooties*, **17**, 256–268.
- Khurana D, Leibson PJ (2003) Regulation of lymphocyte-mediated killing by GTP-binding proteins. *Journal of Leukocyte Biology*, **73**, 333–338.
- Lai WC, Pakes SP, Lu YS, Brayton CF (1987) *Mycoplasma pulmonis* infection augments natural-killer cell-activity in mice. *Laboratory Animal Science*, **37**, 299–303.
- Lai WC, Bennett M, Pakes SP et al. (1990) Resistance to *Mycoplasma pulmonis* mediated by activated natural-killer cells. *Journal of Infectious Diseases*, **161**, 1269–1275.
- Ledford JG, Pastva AM, Wright JR (2010) Collectins link innate and adaptive immunity in allergic airway disease. *Innate Immunity*, **16**, 183–190.
- Ley DH (2008) *Mycoplasma gallisepticum* infection. In: *Diseases of Poultry* (ed. Saif YM), pp. 722–743. Iowa State Press, Ames, Iowa.
- Ley DH, Berkhoff JE, McLaren JM (1996) Isolation of *Mycoplasma gallisepticum* in house finches with conjunctivitis. *Avian Diseases*, **40**, 480–483.
- Liu T, He S-H, Zheng P-Y et al. (2007) Staphylococcal enterotoxin B increases TIM4 expression in human dendritic cells that drives naive CD4 T cells to differentiate into Th2 cells. *Molecular Immunology*, **44**, 3580–3587.
- Luttrell MP, Fischer JR, Stallknecht DE, Kleven SH (1996) Field investigation of *Mycoplasma gallisepticum* infections in house finches (*Carpodacus mexicanus*) from Maryland and Georgia. *Avian Diseases*, **40**, 335–341.
- Luttrell MP, Stallknecht DE, Fischer JR, Sewell CT, Kleven SH (1998) Natural *Mycoplasma gallisepticum* infection in a captive flock of house finches. *Journal of Wildlife Diseases*, **34**, 289–296.
- Manceau M, Domingues VS, Mallarino R, Hoekstra HE (2011) The developmental role of Agouti in color pattern evolution. *Science*, **331**, 1062–1065.
- Markham PF, Glew MD, Browning GF, Whithear KG, Walker ID (1998) Expression of two members of the pMGA gene family of *Mycoplasma gallisepticum* oscillates and is influenced by pMGA-specific antibodies. *Infection and Immunity*, **66**, 2845–2853.
- Marquis JF, Nantel A, LaCourse R et al. (2008) Fibrotic response as a distinguishing feature of resistance and susceptibility to pulmonary infection with *Mycobacterium tuberculosis* in mice. *Infection and Immunity*, **76**, 78–88.
- Marshall ID, Fenner F (1958) Studies in the epidemiology of infectious myxomatosis of rabbits. V. Changes in the innate resistance of wild rabbits between 1951 and 1959. *Journal of Hygiene*, **56**, 288–302.
- Marshall AJ, Miles RJ, Richards L (1995) The phagocytosis of mycoplasmas. *Journal of Medical Microbiology*, **43**, 239–250.
- Matsuo K, Kuniyasu C, Yamada S, Susumi S, Yamamoto S (1978) Suppression of immuno-responses to *Hemophilus gallinarum* with nonviable *Mycoplasma gallisepticum* in chickens. *Avian Diseases*, **22**, 552–561.
- Matute JD, Arias AA, Wright NAM et al. (2009) A new genetic subgroup of chronic granulomatous disease with autosomal recessive mutations in p40(phox) and selective defects in neutrophil NADPH oxidase activity. *Blood*, **114**, 3309–3315.
- Mebius RE, Kraal G (2005) Structure and function of the spleen. *Nature Reviews Immunology*, **5**, 606–616.
- Mohammed J, Frasca S, Cecchini K et al. (2007) Chemokine and cytokine gene expression profiles in chickens inoculated with *Mycoplasma gallisepticum* strains R-low or GT5. *Vaccine*, **25**, 8611–8621.
- Morley SC, Wang C, Lo W-L et al. (2010) The actin-bundling protein L-plastin dissociates CCR7 proximal signaling from CCR7-induced motility. *Journal of Immunology*, **184**, 3628–3638.
- Naylor CJ, Alankari AR, Alafaleq AI, Bradbury JM, Jones RC (1992) Exacerbation of *Mycoplasma gallisepticum* infection in turkeys by rhinotracheitis virus. *Avian Pathology*, **21**, 295–305.
- Nolan PM, Hill GE, Stoehr AM (1998) Sex, size, and plumage redness predict house finch survival in an epidemic. *Proceedings of the Royal Society of London Series B-Biological Sciences*, **265**, 961–965.
- Noller AC, McEllistrem MC, Stine OC et al. (2003) Multilocus sequence typing reveals a lack of diversity among *Escherichia coli* O157: H7 isolates that are distinct by pulsed-field gel electrophoresis. *Journal of Clinical Microbiology*, **41**, 675–679.
- Nordberg J, Arner ESJ (2001) Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. *Free Radical Biology and Medicine*, **31**, 1287–1312.
- Pazdrak K, Young TW, Straub C, Stafford S, Kurosky A (2011) Priming of eosinophils by GM-CSF is mediated by protein kinase C beta II-phosphorylated L-plastin. *Journal of Immunology*, **186**, 6485–6496.
- Peng XJ, Wood CL, Blalock EM et al. (2003) Statistical implications of pooling RNA samples for microarray experiments. *BMC Bioinformatics*, **4**, 26.
- Piertney SB, Oliver MK (2006) The evolutionary ecology of the major histocompatibility complex. *Heredity*, **96**, 7–21.
- Raida MK, Buchmann K (2008) Development of adaptive immunity in rainbow trout, *Oncorhynchus mykiss* (Walbaum) surviving an infection with *Yersinia ruckeri*. *Fish & Shellfish Immunology*, **25**, 533–541.
- Razin S, Yoge D, Naot Y (1998) Molecular biology and pathogenicity of mycoplasmas. *Microbiology and Molecular Biology Reviews*, **62**, 1094–1156.
- Roberts SR, Nolan PM, Hill GE (2001a) Characterization of *Mycoplasma gallisepticum* infection in captive house finches (*Carpodacus mexicanus*) in 1998. *Avian Diseases*, **45**, 70–75.
- Roberts SR, Nolan PM, Lauerman LH, Li LQ, Hill GE (2001b) Characterization of the mycoplasmal conjunctivitis epizootic in a house finch population in the southeastern USA. *Journal of Wildlife Diseases*, **37**, 82–88.
- Samstag Y, Eibert SM, Klemke M, Wabnitz GH (2003) Actin cytoskeletal dynamics in T lymphocyte activation and migration. *Journal of Leukocyte Biology*, **73**, 30–48.
- Sarson AJ, Parviz P, Lepp D, Quinton M, Sharif S (2008) Transcriptional analysis of host responses to Marek's disease

- virus infection in genetically resistant and susceptible chickens. *Animal Genetics*, **39**, 232–240.
- Scheele JS, Marks RE, Boss GR (2007) Signaling by small GTPases in the immune system. *Immunological Reviews*, **218**, 92–101.
- Shapiro MD, Marks ME, Peichel CL et al. (2004) Genetic and developmental basis of evolutionary pelvic reduction in threespine sticklebacks. *Nature*, **428**, 717–723.
- Soley JT, Roberts DC (1994) Ultrastructure of ostrich (*Struthio camelus*) spermatozoa. II. Scanning electron microscopy. *Onderstepoort Journal of Veterinary Research*, **61**, 239–246.
- Sommer S (2005) The importance of immune gene variability (MHC) in evolutionary ecology and conservation. *Frontiers in Zoology*, **2**, 16.
- Spurgin LG, Richardson DS (2010) How pathogens drive genetic diversity: MHC, mechanisms and misunderstandings. *Proceedings of the Royal Society of London Series B-Biological Sciences*, **277**, 979–988.
- Stumpfner-Cuvelette P, Benaroch P (2002) Multiple roles of the invariant chain in MHC class II function. *Biochimica Et Biophysica Acta-Molecular Cell Research*, **1542**, 1–13.
- Todd EM, Deady LE, Morley SC (2011) The actin-bundling protein L-plastin is essential for marginal zone B cell development. *Journal of Immunology*, **187**, 3015–3025.
- Toomey MB, Butler MW, McGraw KJ (2010) Immune-system activation depletes retinal carotenoids in house finches (*Carpodacus mexicanus*). *Journal of Experimental Biology*, **213**, 1709–1716.
- van de Wetering JK, van Golde LMG, Batenburg JJ (2004) Collectins – players of the innate immune system. *European Journal of Biochemistry*, **271**, 1229–1249.
- van der Most PJ, de Jong B, Parmentier HK, Verhulst S (2011) Trade-off between growth and immune function: a meta-analysis of selection experiments. *Functional Ecology*, **25**, 74–80.
- van der Sar AM, Spaink HP, Zakrzewska A, Bitter W, Meijer AH (2009) Specificity of the zebrafish host transcriptome response to acute and chronic mycobacterial infection and the role of innate and adaptive immune components. *Molecular Immunology*, **46**, 2317–2332.
- Wang JB, Nygaard V, Smith-Sorensen B, Hovig E, Myklebost O (2002) MArray: analysing single, replicated or reversed microarray experiments. *Bioinformatics*, **18**, 1139–1140.
- Wang Z, Farmer K, Hill G, Edwards SV (2006) A cDNA macroarray approach to parasite-induced gene expression in a songbird host: genetic response of House Finches to experimental infection by *Mycoplasma gallisepticum*. *Molecular Ecology*, **15**, 1263–1273.
- Wang C, Morley SC, Donermeyer D et al. (2010) Actin-bundling protein L-plastin regulates T cell activation. *Journal of Immunology*, **185**, 7487–7497.
- Whitcomb RF (1983) Culture media for spiroplasmas. In: *Methods in Mycoplasmology* (eds Razin S, Tully JG), pp. 147–158. Academic Press, New York City, New York.
- Wright S (1955) Classification of the factors of evolution. *Cold Spring Harbor Symposia on Quantitative Biology*, **20**, 16–24.
- Yang YH, Speed T (2002) Design issues for cDNA microarray experiments. *Nature Reviews Genetics*, **3**, 579–588.
- 
- The authors study host-parasite interactions to understand the evolutionary consequences of disease outbreak (CB and SVE), and how they relate to the evolution of ornamental traits in vertebrates (SLB and GEH). JZ develops integrated Systems Biology approaches to understand the etiology of aging and diseases.
- 
- ### Data accessibility
- Sequence data are deposited on NCBI GenBank with accession numbers GW346076–GW346170; the microarray data have been deposited in NCBI's Gene Expression Omnibus (Edgar et al. 2002) and are accessible through GEO Series accession number GSE35931 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE35931>).
- ### Supporting information
- Additional supporting information may be found in the online version of this article.
- Data S1** Gene function.
- Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting information supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.