Evolution of Pathogen-Induced Gene Expression in the House Finch, *Carpodacus mexicanus*

by

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Abstract

Bacterial pathogens impact the survival, reproduction and fitness of their hosts making them extremely well-suited for experimental studies intended to understand adaptive phenotypic evolution of wild populations of vertebrate hosts. Pathogen-driven host phenotypic changes may occur from non-genetic effects like developmental plasticity or maternal effects, or from adaptive evolutionary processes like natural or sexual selection. When infectious diseases emerge and spread rapidly, they can have major consequences on host population dynamics, potentially resulting in rapid evolution of host phenotypes. The outbreak and spread of the pathogenic bacterium *Mycoplasma gallisepticum* (MG) in house finches (*Carpodacus mexicanus*) represents an excellent system for studying the evolution of such adaptive phenotypes in the wild. House finches are passerine songbirds ranging naturally across most of western North America. They were introduced to New York in 1940 and have since expanded throughout the entire eastern US. Male house finch ornamental plumage color is an important criterion in female mate choice, and infection with pathogens during molt can have significant effects on color expression. MG is a common pathogen of poultry that causes upper respiratory and eye conjunctivitis infections. It was first detected in house finches in the Washington, D.C. metro area in 1994, after which it spread across the entire eastern population in just a few years. MG had not, however, reached populations of the US Southwest when the infection experiments described hereafter were conducted.

In this dissertation I use experimental and molecular approaches to investigate and identify molecular candidates associated with house finch phenotypic evolution, as driven by history of infection with MG. I develop a custom cDNA microarray designed to study the expression of genes in house finches infected with MG. I then derive a set of candidate genes from results of microarray studies and develop a highly multiplexed qRT-PCR assay for house finch immune tissues. Using these tools, I show that population-level phenotypic differences in resistance are associated with differences in gene expression in the spleen. Furthermore, expression responses of western US birds to experimental infection were more similar to eastern US birds studied in 2000 than to eastern birds in 2007. These results support the hypothesis that resistance has evolved in eastern birds over only 12 years. I further show that phenotypic differences in plumage color are also associated with differences in gene expression in both the spleen and the trachea. Together, these results contribute to our understanding of the potential for rapid vertebrate phenotypic evolution when populations are under strong selective pressures by pathogens, and they provide a basis for the continued study of gene expression in house finches as evidence of evolution by natural and sexual selection.

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I. RAPID EVOLUTION OF DISEASE RESISTANCE IS ACCOMPANIED BY FUNCTIONAL CHANGES IN GENE EXPRESSION IN A WILD BIRD

Abstract

Wild organisms are under pressure to rapidly adapt to environmental changes. Predicting the impact of these changes on natural populations requires an understanding of the speed with which adaptive phenotypes can arise and spread, as well as the underlying mechanisms. However, our understanding of these parameters is poor in wild animals. Here I use experimental and molecular approaches to investigate the recent emergence of resistance in North American house finches (*Carpodacus mexicanus*) to *Mycoplasma galliseptum* (MG), a severe conjunctivitis-causing bacterium. Two-weeks following experimental infection, finches from eastern U.S. populations with a 12-year history of exposure to MG harbored approximately one-third fewer MG in their conjunctivae than finches from western U.S. populations with no prior exposure to MG. Using a cDNA microarray, I show that this phenotypic difference in resistance was associated with differences in splenic gene expression, with finches from the exposed populations up-regulating immune genes post-infection and those from the unexposed populations generally down-regulating them. The expression response of western U.S. birds to experimental infection was more similar to eastern U.S. birds studied in 2000, seven years earlier in the epizootic, than to eastern birds in 2007. These results support the hypothesis that resistance has evolved in the exposed populations over the 12 years of the epizootic. I hypothesize that host resistance arose and spread from standing genetic variation in the eastern U.S., and highlight that

natural selection can lead to rapid phenotypic evolution in populations when acting on such variation.

Introduction

A pressing question in modern biology is how quickly natural populations can respond to anthropogenic selection pressures (Walther et al. 2002). Integral to predicting evolvability will be an understanding of the speed with which adaptive phenotypes can spread in a population and their underlying molecular bases. While adaptive phenotypic changes have been suggested to evolve rapidly in wild animals, sometimes within a few generations (Reznick et al. 1997, Grant & Grant 2006), few studies confirm that such changes arise through the selection of adaptive genotypes rather than from plastic consequences of gene by environment (G*E) interactions (Badyaev 2008). Although there is growing appreciation that phenotypic plasticity might play an important role in the evolution and spread of adaptive phenotypes, ultimately evolution requires adaptive changes in gene frequencies (Fisher 1958, Falconer & Mackay 1996). Studies that have been able to tease evidence of evolution from other mechanisms of phenotypic change not only provide some of the most convincing evidence of evolution by natural selection, but also enhance our understanding of evolvability and its underlying processes (Ferea et al 1999, Blount et al. 2008). However, identifying beneficial mutations and measuring their spread in response to a selective agent is not straightforward (Barrett et al. 2008, Blount et al. 2008, Linnen et al. 2009). One alternative is to identify evidence of evolutionary change and then measure phenotypic and accompanying molecular changes within an experimental framework designed to rule out other sources of influence.

Host-parasite systems represent dynamic interactions and so provide outstanding models for studying evolutionary change (May & Anderson 1983, Decaestecker et al. 2007). For example, when parasites represent novel and intense selection pressures, it is possible to document the spread of host resistance within populations over just a few generations (Best & Kerr 2000), as has been recorded recently in eastern U.S. house finches (*Carpodacus mexicanus*) (Hochachka & Dhondt 2000, Roberts et al. 2001, Farmer et al. 2002). House finches are native to western North America, but in 1940 a founder population was introduced to the eastern U.S., near New York City. By 1990, house finches had spread throughout much of eastern North America and numbered over 100 million, although they remained geographically isolated from their western counter-parts (Hill 1993). In 1994, *Mycoplasma gallisepticum* (MG), a bacterium found in poultry (Stipkovits & Kempf 1996) but not in songbirds (Ley et al. 1997), was detected in the eastern populations of house finches (Ley et al. 1996). MG causes respiratory tract and conjunctivitis infections, and as a result, many millions of eastern house finches died between 1994 and 1998 (Hochachka & Dhondt 2000). Naturally-infected captive finches confirmed mortality due to MG-induced conjunctivitis (Luttrell et al. 1998; Fig. 1a). However, declines in eastern populations began stabilizing in 1998/99 (Hochachka & Dhondt 2000), experimental infections of 1999-hatched finches in Alabama showed precursory evidence of resistance (Roberts et al. 2001, Farmer et al. 2002), and evidence from this 2007-study shows MG-conjunctivitis reduced to endemic levels (see Methods).

MG is known for its ability to manipulate host immunity in poultry (Ganapathy & Bradbury 2003, Mohammed et al. 2007). At the onset of infection, MG triggers the up-regulation of pro-inflammatory cytokines and chemokines (Gaunson et al. 2000, Mohammed et al. 2007), and induces a non-specific inflammatory response (Gaunson et al. 2006), which can cause damage to

host epithelia (Ley 2008). However, MG has also been found to have immunosuppressive effects (Mohammed et al. 2007), particularly during later stages of the immune response (Gaunson et al. 2000). For example, 1-2 weeks post-infection, MG has shown to be associated with a decline in the infiltration of T cells in the trachea (Gaunson et al. 2000) and a suppression of T-cell activity (Ganapathy & Bradbury 2003) in chicken (*Gallus gallus domesticus*). There is also evidence that infection with MG is associated with a reduced humoral antibody response to other pathogens (Matsuo et al. 1978, Naylor et al. 1992). These immunomodulatory properties, be they induced directly or indirectly by MG, allow MG to evade and suppress host defenses (Razin et al. 1998).

Changes in disease dynamics in eastern house finches appear to provide evidence of rapid evolution of host resistance, but two other factors could drive the apparent emergence of resistance: (i) phenotypic plasticity (including acquired immunity, maternal effects and short/long-term condition-dependent effects); and (ii) attenuation of MG virulence. The aim of this study was to use experimental and molecular approaches in the above host-parasite system to test the basis for the emergence of resistance. I conducted an MG-infection experiment and compared MG-loads and gene expression profiles 2-weeks post-inoculation in birds captured in 2007 from eastern (Alabama) and western (Arizona) U.S. populations. I also compared these results to expression profiles previously published from a similar experiment conducted on birds captured in 2000 in Alabama (Wang et al. 2006). MG was first detected in Alabama in 1995 (Nolan et al. 1998), but had never been reported in Arizona prior to 2009, despite long-term monitoring (Dhondt et al. 2006, Toomey et al. 2010). Alabama and Arizona are on similar latitude and sampling was conducted at 3 different suburban sites in both States. In Arizona, the sites were 1-2 km apart and the birds were captured over 3 days (hereafter: Arizona population), while in Alabama, the sites were 10-103 km apart and the birds were captured over the course of

a month (hereafter: Alabama population). Birds were kept in identical conditions on *ad libitum* food and water for 3 months before the onset of the experiment. All 2007 experimental birds were inoculated with the same January 2007 Alabama strain of MG (see Methods). The 2000 study was conducted using birds from the same Alabama population as the current study but with a 1999 strain of MG (Wang et al. 2006), and took place before the spread of resistance in Alabama (Roberts et al. 2001, Farmer et al. 2002).

None of the birds I used from either population had been exposed to MG during their lifetimes, as confirmed by both PCR and agglutination assays (see Methods). This status removes inter-population differences in responses to infection caused by immune priming from prior exposure to MG. First, I tested whether birds from Alabama and Arizona in 2007 differed in their level of resistance to MG by quantifying MG-load in the conjunctivae of birds 2-weeks post-infection. Second, I assessed how birds from Alabama and Arizona differed in their response to infection by quantifying changes in gene expression, again after 2 weeks. Third, I investigated how molecular responses to infection have changed over time by conducting a quantitative comparison of gene expression differences between birds captured from Arizona in 2007 and from Alabama both in 2000 and 2007 (Wang et al. 2006).

Materials and Methods

Capture, housing and experimental infection

Birds were captured at the two sites using mist nets or wire mesh cages placed around feeders. 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. Birds were

housed in cages as pairs for the duration of their period in captivity. Cages were kept indoors, in temperature controlled rooms with windows, and birds were fed sunflower seed, brown and white millet, and water *ad libitum*, as well as apple slices and crushed eggshells on a weekly basis. Grit was provided to allow digestion of seeds. Birds from Alabama and Arizona were kept in separate rooms for the first month to check that birds were MG-free. Following quarantine, birds were measured and blood sampled using brachial venipuncture (~60 µl of whole blood) and examined for exposure to MG using serum plate agglutination assay (SPA) (Luttrell et al. 1998) and amplification of MG DNA from choanal and conjunctival swabs (Roberts et al. 2001). No birds used in any part of the experiment were found to have been previously infected with MG. Twelve birds from the Alabama population were removed from the experiment following evidence of exposure to MG (8 were symptomatic at capture, 1 developed symptoms during quarantine and 3 were seropositive for MG-antibodies), and 15 from Arizona and 32 from Alabama were used in a different experiment, leaving 22 Arizona birds and 21 Alabama birds in this study.

Birds were kept either as controls or infected via ocular inoculation with 20 µl of culture containing $1x10^4$ to $1x10^6$ color changing units/ml of an early 2007 Auburn MG isolate (BUA #243). Control birds were sham infected using sterile SP4 medium (Whitcomb 1983). Control and infected birds were maintained under identical conditions, but in separate rooms of the aviary. After exposure, birds were monitored daily for disease onset and progression of symptoms. All experimentally infected birds tested positive for MG DNA in their choanal cleft 3 and 14 days post-inoculation and all were seropositive after two weeks. All control birds remained negative for MG DNA and for MG antibody agglutination throughout the course of the experiment. Fourteen days post treatment, birds were euthanized under license. The spleens and

the eyes/conjunctivae from all euthanized birds were immediately removed, stored in RNAlater (Ambion) and placed at -80°C.

Quantification of MG using TaqMan quantitative RT-PCR assays

I randomly selected one eye with conjunctivae from each bird 14 days after infection. I isolated total genomic DNA from both MG and house finches using the Qiagen AllPrep DNA/RNA Mini Kit. MG quantification was then performed as described in (Grodio et al. 2008) by running qRT-PCR assays on the *mgc2* gene. I also amplified the house finch *rag1* gene to control for variation in amounts of starting material. Reactions were run on an ABI Prism 7500 (Applied Biosystems). I made a standard curve for both genes using 100, 50, 25, 10, 1 and 0.1 g/l of genomic DNA to estimate the relative amount of MG between individuals. Each reaction consisted of 25ml of TaqMan PCR Master Mix, No AmpErase UNG (Applied Biosystems), 0.45ml each of 100 μM forward and reverse primers, 1.25ml of 10μM probe, 17.85ml of DNase-free water and 5l of 10g/l sample. Cycling parameters were 50°C for 2 min, 95°C for 10 min, followed by 50 cycles for 95°C for 15 sec and 60°C for 1 min. I used the automatic threshold settings for analysis of my samples.

Microarray construction

I constructed a microarray using cDNA clones from the subtraction suppression hybridization (SSH) libraries constructed in Wang et al. (2006) and enriched in cDNA differentially expressed between MG-infected and control house finches 2-weeks post-infection (Wang et al. 2006). The microarray consisted of 1000 unique amplicons and included the 220 clones previously identified as significantly differentially expressed between control and infected house finches using a

macroarray approach (Wang et al. 2006), as well as 694 randomly selected clones from the librairies, many of whose expression responses to infection are unknown (Wang et al. 2006). The clones were collected from the SSH libraries with a toothpick and grown in 1.3 ml of LB + ampicilin at 37°C overnight. I isolated the plasmid DNA using a Plasmid & BAC extraction kit (AutoGen) on an AutoGenprep 965. I amplified 5 µl of the eluted DNA in 10 µl Buffer 10 (Lucigen), 0.8 µl dNTP (100 µM), 8 µl of each primer M13/M13R (10 µM), 0.8 µl (4 U) EconoTaq DNA polymerase (Lucigen) and 56.4 µl of sterile dH_2O. The reaction was run for 35 cycles consisting of a 90s 94°C denaturating step, a 45s 50°C annealing step and a 45s 72°C extension step. PCR products were purified using a QIAGEN MinElute 96 UF Purification kit and run on a 2% agarose gel. Amplified inserts were subsequently printed onto the array slides.

I also printed five house finch 'housekeeping' genes to help in normalization procedures (Actin related protein 2/3, ATP synthetase, ATPase V1 subunit G1, Basic transcription factor 3, Calmodulin 2; accession numbers: bankit1324533, 1324536, 1324538, 1324542, 1324554). These genes were generated by amplification of cDNA extracted from house finch spleens (see below), using degenerate primers designed from conserved sequences of humans, mice, chicken and, when available, zebra finches. I amplified 2 µl of cDNA in 2.5µl Buffer 10 (Lucigen), 2.5 µl dNTP (100 µM), 1 µl of each primer (10 µM), 0.2 µl (2.5U) EconoTaq DNA polymerase (Lucigen) and 15.8 µl of sterile dH_2O. The reaction was run for 35 cycles consisting of a 1 min 94°C denaturating step, a 2 min 60°C annealing step and a 3 min 72°C extension step. PCR products were run on a 2% agarose gel and purified using a QIAGEN MinElute 96 UF Purification kit. Each gene was amplified twice and while I kept one reaction intact, I ligated and transformed the other into One Shot TOP10 Chemically Competent *Escherichia coli* using the TOPO Cloning kit (Invitrogen). Colonies were picked and added to 1.3 ml of LB medium

with ampicilin to grow overnight at 37°C. I then isolated the plasmid DNA using a Plasmid & BAC extraction kit (AutoGen) on an AutoGenprep 965. I amplified 2 µl of the extracted plasmids in 2.5 µl Buffer 10 (Lucigen), 0.2 µl dNTP (100 µM), 2 µl of each primer M13/M13R (10 µM), 0.2 µl (2.5U) EconoTaq DNA polymerase (Lucigen) and 14.1 µl of sterile dH_2O. All PCR products were purified using a QIAGEN MinElute 96 UF Purification kit and run on a 2% agarose gel. I verified the identity of the purified PCR products by sequencing them on an ABI 377 DNA sequencer (see below). I printed both the PCR products and the transformed PCR products of the five house finch housekeeping genes onto the array slides. I quantified purified PCR products on a Nanodrop spectrophotometer.

Additionally, I printed the PCR products from the DNA amplification of 11 *E. coli* housekeeping genes (arcA, aroE, dnaE, gapA, gnd, icdA, pgm, polB, putin, trpA, trpB) to serve as external spike-ins (van Bakel & Holstege 2004). DNA was extracted from a few *E. coli* colonies obtained above using the QIAGEN DNeasy Tissue and I amplified *E. coli* house keeping genes as described in (Noller et al. 2003, Hommais et al. 2005). PCR products were purified, sequenced and quantified as described above.

I printed the clones on poly-L-lysine coated glass slides using an OmniGrid 100 (GeneMachines, BST Scientific). All clones were printed twice on each grid and each grid was replicated twice on each microarray slide. After printing, the slides were blocked by rehydration, UV crosslinking at 60 mJ, and dipped in blocking solution (6g Succinic Anhydride, 335 ml 1-Methyl-2-pyrrolidinone, 15 ml Sodium Borate). Denaturing was subsequently performed by dipping the slides in MiliQ water, and then in 95% Ethanol, and spun dry by centrifugation for 2 min at 1000 rpm. The desiccated slides were stored at room temperature in a closed container until further use.

Sample preparation and microarray hybridization

The slides were hybridised with the samples collected from the experimental infections. I 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' protocols. Gene expression changes were examined in the spleen because this tissue plays an important role in the organization of both the innate and the acquired immune responses in humans as well as in birds, in addition to its role of filtering blood and removing old erythrocytes (Mebius & Kraal 2005, Davison et al. 2008). Indeed, asplenic human infants are found to be extremely sensitive to bacterial infections and the lack of a spleen results in defective Th cells, decreased antibody responses and a lack of important macrophages (Borek 1986, Brendolan et al. 2007). I 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 -80ºC until further processing.

I pooled 2 to 5 spleens from birds from the same population in the same treatment to generate enough mRNA for microarray hybridizations. I had 2 samples for each treatment from each population. Samples were labelled using Cy5 dye and were then hybridised against a common reference, made by pooling an aliquot of all the individual samples and labelled with Cy3. I made a calibration curve of hybridization efficiency by diluting the *E. coli* external PCR products at known concentrations. Each hybridization was performed on one half-slide. Each pooled House finch RNA sample was prepared for cDNA microarray hybridization by reverse-transcribing 15µg of total pooled RNA in 30.8 µl reaction volumes containing 1 µl of a mix of oligo dT (dT12-, 13-, 14-, 15-, 16-, 17- and 18-mer; final concentration 5 µg/µl), 6 µl of 5 First

Strand Buffer (Invitrogen), 3 µl of 0.1M DTT, 0.8 µl 50 aminoallyl-dUTP/dNTP mix (20mM dATP, 20mM dCTP, 20mM dGTP, 12mM dTTP, 8mM aminoallyl-dUTP), 2.5 µl (500U) of Superscript II Reverse Transcriptase (Invitrogen). Reactions were incubated at 42ºC for 3h. cDNA samples were subsequently hydrolyzed by adding 10 µl of 1M NaOH, 10 µl of 0.5M EDTA and incubated at 65ºC for 15 min. Neutralization was performed by addition of 25 µl 1M HEPES pH 7.5.

I prepared the external spike-ins by dividing the product from each *E.coli* gene amplification into two. I incorporated the aminoallyl dUTP by adding 24 µl of nuclease free H_2O to 1 µg of each sample. I also added 20 µl of BioPrime 2.5 random primer mix (Invitrogen) and boiled the reaction for 5 min before incorporating 5 µl of 10 aminoallyl-dUTP/dNTP mix (4.8 mM dATP, 4.8 mM dCTP, 4.8 mM dGTP, 1.6 mM dTTP, 3.2 mM aminoallyl-dUTP) and1 µl (40-50U) of Klenow Fragment to each tube. I incubated at 37ºC for 2h and stopped the reaction with 5 µl 0.5M EDTA. For each *E. coli* gene, the labelled samples were serially diluted and I added pools of the more concentrated products to the common house finch reference (final concentrations: polB and dnaE at 182 pg/µl, trpA and putin at 91 pg/µl, pgm and arcA at 18.2 pg/µl, gnd and gapA at 3.64 pg/µl, trpB and icdA at 1.82 pg/µl), and a pool of diluted products to the each of the house finch labelled cDNA samples (all samples added at a final concentration of 1.82 pg/µl), to obtain final concentration ratios of 1:1, 1:2, 1:10, 1:50, and 1:100.

I purified the mixtures using microcon-30 filters. I added 1 µl of $NaHCO_3$ to 10 µl purified probe. I resuspended the cyanine dyes (Cy3 and Cy5) with the probe and incubated the mixture in the dark at room temperature for 2h to allow coupling of the cDNA to the N-hydroxy succinamide ester of the dyes. All samples were labelled using Cy5 dye and the common reference was labelled with Cy3. The mixtures were then purified using QIAGEN QIAquick

PCR purification kit. The eluted Cy-3 and Cy-5 labeled probes were combined and added into a microcon-30 filter, spun for 3 min at 10000 g and the filter was then inverted and spun at 4500 g for 5 min. I prepared the concentrated probes for hybridization by adding 3 µl of 20 SSC, 1.5 µl poly(A) (7 µg/µl), 0.48 µl 1M HEPES pH 7.0, and I applied the mixture to pre-humidified Millipore 0.45 µm filters, spun them at 10,000 g for 1 min and stored the flow-through at 4ºC until further use.

Immediately prior to hybridization, I added 0.45 µl 10% SDS to the probe, heated it for 2 min at 100ºC and allowed it to cool down at room temperature for 10 min. The arrays were placed in Corning Microarray hybridization chambers and a clean Lifterslip (Eerie Scientific) was placed over each array. After injecting the probes under the lifterslips, I added 50 µl 3 SSC to the hybridization chambers and placed them in a 62ºC water bath for 12-16h. Following hybridization, the slides were washed in 0.2 SSC with 2% SDS and then in 0.2 SSC. I spun the slides dry by centrifuging them at 1000 rpm for 2 min and scanned using an Axon 4000A microarray scanner (Axon Instruments).

Statistical analysis of microarray data

I used the software package GenePix to yield log base-2 ($\log_2$) measurements for mean fluorescence intensities for each dye channel in each spot on the array and to flag low quality spots. Normalisation of the raw fluorescence intensities was performed in 3 steps using bioconductor package marray and limma (Smyth 2004) implemented in R language (http://www.r-project.org). First, background adjustment was performed using the normexp method. Second, spatial and print-tip loess normalisation (two dimensional method) were

performed to remove spatial and dye biases for each slide. Third, I performed a scale normalisation to control for variation between slides.

The ratios generated by the external spike-ins were used for quality control. To control for within-hybridization spatial variation, I compared the signal from the 2 replicated grids. To control for between-slide differences, I compared the signals from the *E. coli* external spike-ins, the house finch housekeeping genes and the common reference on the different slides. All clones were printed twice on each array and were considered to be differentially expressed only when both replicates displayed a significant deviation from the mean of the standard.

To determine gene expression differences between samples, normalized $\log_2$ transformed signal ratio (sample versus reference) were fitted to a general linear model with two factors representing treatment (control and infected) and population of origin (MG-exposed Alabama and MG-unexposed Arizona) and of the form:

$$Y_{ijc} = \mu + A_i + B_j + AB_{ij} + \varepsilon_{ijc}$$

where Y_{ijc} is the $\log_2$ measurement for a particular clone (c) from a particular treatment (i) and a particular population of origin (j), μ is the parametric mean, A and B correspond to the single factor effects (treatment and population of origin, respectively), AB is the two-way interaction between the two main effects, and ε is the residual between the data and the model.

I identified clones that were significantly differentially expressed between the following groups: (1) infected *vs.* controls in the MG-unexposed population; (2) infected *vs.* controls in the MG-exposed population; (3) control birds from the unexposed population *vs.* control birds from the exposed population and (4) infected birds from the unexposed population *vs.* infected birds from the exposed population. These comparisons allowed me to evaluate changes in gene

expression between treatments within geographic populations, as well as within treatments between populations.

Sequencing and gene ontology analyses

All the 162 clones found to be significantly differentially expressed between groups were subsequently sequenced. I added 1 µl of Big Dye (Applied Biosystems), 2 µl of Buffer 5 and 2 µl of primer M13 or M13R (1µM) to 5 µl of PCR product. The reaction was run for 30 cycles consisting of 10s at 94ºC, 5s at 50ºC and 4 min at 60ºC, and a final extension of 1 min at 60ºC. The sequencing reaction cleanup was performed by adding 2.5 µl of 125mM EDTA to each reaction, 25 µl 95-100% ethanol, incubating for 15 min at room temperature and centrifuging at 3000 for 30 min. The tubes were then inverted, centrifuged at 190 g and I added 30 µl of 70% ethanol, centrifuged for 15 min at 1650 g, then inverted and spun at 190 g for 1 min. The cleansed sequence reaction pellet was resuspended with 10 µl of HiDi-Formamide before sequencing on an ABI 377 sequencer. Forward and reverse sequences generating a BLAST hit with an e-value $< 1\times10^{-20}$ with more than 100 nucleotides were categorized by their vertebrate homologues, while all other genes were considered to be unknown. Gene ontology category and function were determined using Harvester (http://harvester.fzk.de/harvester/).

Microarray validation using multiplex quantitative RT-PCR analysis

I verified transcriptional changes at 16 genes using multiplex quantitative real-time amplifications (Hembruff et al. 2005, Chapter 3). Multiplex qRT-PCRs require only a small amount of RNA to simultaneously assess individual variation in expression of up to 30 different genes per sample. Genes were selected if they were significantly differentially expressed in the

microarray experiment and if they were of known, particularly immune-related, GO functions (immunoglobulin J, parathymosin, MHC class II-associated invariant chain Ii, immunoglobulin superfamily member 4A isoform a, TCR beta chain, hsp90, NADH dehydrogenase subunit 4, thioredoxin, prosaposin, eukaryotic translation initiation factor eIF4E, nucleic acid binding protein RY-1 variant 3, MAK-like kinase, RhoA GTPase, ubiquitin C, lymphocyte cytosolic protein, SEC61 gamma subunit) for inclusion in a single multiplex. I also included two house finch housekeeping genes (Actin related protein 2/3 and Calmodulin 2) that were used in the microarray experiment to help in the normalization of qRT-PCR results.

Primers were designed using GeXP Express Profiler Primer Design software (Beckman, Fullerton, CA). Each primer pair was designed to yield PCR products at least 4 bp apart (ranging from 139 to 341 bp) with similar GC content and melting temperature. I also included primers to amplify a kanamycin RNA transcript that was spiked into each reaction as an external control (GenomeLab GeXP Start Kit, Beckman Coulter). Multiplex qRT-PCR maintains relative transcript abundances through incorporation of universal tags that are homologous to the 5' ends of the forward and reverse primers. The forward universal primer carries a fluorescent dye label so that, following amplification, the PCR products can be examined by capillary electrophoresis (Beckman Coulter CEQ8000) for fragment size determination.

Two contrasting analytical approaches were used to validate microarray data. First, I used a correlational approach to determine whether the degree to which genes are differentially expressed in the microarray is related with the degree to which they are so in a multiplex quantitative RT-PCR. Differential expression of genes in the microarray is defined as those in which the expression of the same gene differs significantly either between treatments within the same population or between populations within treatments. As predicted, a Spearman's rank

correlation showed a significant positive relationship between the ranks of expression from the microarray and the multiplex qRT-PCR data (r_s=0.46, *N*=45, *P*<0.001). Second, I used a one-sampling T-test framework to determine whether up-regulation of expression in the microarray is associated with up-regulation using quantitative RT-PCR, and *vice versa*. Two analyses were conducted using the following comparisons: (i) control birds from MG-unexposed *vs.* MG-exposed population (*N*=11 genes); and (ii) infected birds from MG-unexposed *vs.* MG-exposed population (*N*=16). In both analyses, up-regulation in the microarray was associated with up-regulation in the multiplex qRT-PCR (and *vice versa*): (i) T_{10}=2.26 *P*=0.024; (ii) T_{15} = 3.33, *P*=0.002). (*P* values represent one-tailed estimates, since in all cases the microarray is only upheld if the qRT-PCR results are greater than zero).

Statistical analyses

The validity of all further analyses was confirmed using Zar (Zar 2007). Microarray validation - A spearman's rank correlation was conducted because neither the microarray nor multiplex qRT-PCR values were normally distributed (Shapiro-Wilk test for normality: *W*=0.93, *N*=44 *P*=0.007 (microarray); *W*=0.68, *N*=44, *P*<0.001 (qPCR)). T-tests were conducted because inter-population differences in gene expression between control and infected did not differ from normality (Shapiro-Wilk test for normality: *W*=0.95, *N*=11, *P*=0.69 (microarray); *W*=0.91, *N*=16, *P*=0.12 (qPCR)). Inter-population/treatment comparisons (see Fig. 1b) - Such differences were analyzed using two-tailed Binomial tests, Fisher exact tests or Goodness-of-fit tests. Two-tailed binomial tests resemble 2x2 chi-squared contingency tables and give qualitatively similar results except that they are more appropriate when comparing between 2 proportions. Fisher exact tests were used when sample sizes were such that expected values failed to reach 5. Goodness-of-fit

tests were used to compare whether an observed frequency differed from expected. MG Analyses - Population differences in MG were analyzed using a General Linear Model with normal error structure in which MG-load was fitted as the response term, amount of host-tissue was fitted as a co-variate and population was fitted as the main term of interest. The distribution of MG-loads did not differ significantly from normality (Shairo-Wilk test for normality; W=0.97, P=0.74) and the variance in MG-load between populations did not differ significantly (Bartlett's test for homogeneity of variance; χ_1^2=0.00, P=0.96). The relationship between MG-load and expression levels was conducted using a Linear Regression. MG-load was expressed as a ratio of host-tissue and used as the response term in the analysis. The distribution of MG-load ratio did not differ from normality (Shapiro-Wilk test; W=0.94, P=0.22).

Results

(1) Population differences in MG-load

If MG-loads are lower in birds from Alabama than Arizona following maintenance in identical *ad libitum* conditions for 3 months and infection with the same strain of MG, this would support the hypothesis of rapid evolution of resistance. In addition, it would rule out the possibility that emergence of resistance in Alabama resulted solely from: (a) short-term environmental effects, such as improvements to individual body condition; or (b) reductions in the virulence of MG. After controlling for the confounding influence of the amount of host tissue sampled (General Linear Model (GLM): $F_{1,21}$ = 9.41, P = 0.006), I found that MG-load differed significantly between populations ($F_{1,21}$ = 13.0, P = 0.002, R^2 = 30%) (Fig. 1b). Birds from Alabama in 2007 already showed a 33% reduction in MG-load in their conjunctiva 2 weeks after experimental

infection compared with birds from Arizona in 2007, a substantial difference given that mortality as a result of MG usually occurs 25-70 days after the onset of conjunctivitis (Hill 2002). These results support the hypothesis that birds have evolved resistance to MG in Alabama, but it is not yet possible to rule out a confounding influence of long-term (life-long) differences in individual body condition.

(2) Population differences in gene expression patterns

Investigating patterns of gene expressions following infection can elucidate mechanisms of MG infection and host responses. Transcript levels were quantified using a microarray printed with cDNA clones selected from two subtraction suppression hybridization libraries enriched in clones differentially expressed between MG-infected and control house finches 2-weeks post-infection (see Methods). Differences in transcript levels were tested between: infected *vs.* control birds in Arizona in 2007 (Comparison 1); infected *vs.* controls in Alabama in 2007 (Comparison 2); control birds from Arizona *vs.* Alabama in 2007 (Comparison 3) and infected birds from Arizona *vs.* Alabama in 2007 (Comparison 4) (see Fig. 2a). Overall, after correcting for false discovery rate, 162 clones were found to be significantly differentially expressed; sequencing and blast searches in GenBank for vertebrate homologues revealed a subset of 52 genes of known function that were differentially expressed across at least one of these four comparisons (Fig. 2b; see Appendix 1 and Table 1 for details on gene functions).

Birds from the two populations in 2007 showed significant differences in both the number and direction of expression changes following infection. First, a greater percentage of the 52 genes of known function showed post-infection expression changes in Alabama (38%) than in Arizona (21%) (Comparison 1 *vs.* 2; two-sample *Binomial test* = 1.93, $P = 0.05$). This difference

was generated by a greater percentage of genes being down-regulated in Arizona than in Alabama (80% of 20 *vs*. 27% of 11 genes; *Fisher exact test, P* = 0.007). Second, while 67% of the 52 genes were differentially expressed between control birds of the two populations (Comparison 3), this increased to all 52 genes being differentially expressed between experimental birds of the two populations (Comparison 4), representing a significant increase in between-population expression differences following infection (two-sample *Binomial test* = -4.51, $P < 0.001$). Again, this difference was generated by a greater percentage of genes being expressed at lower levels in Arizona versus Alabama (90 *vs*. 10%; two-sample *Binomial test* = 8.24, $P < 0.001$).

The results above are largely driven by differential expression of functionally relevant immune genes. Of the 52 genes showing differential expression in at least one of the four comparisons above (Figs. 2*A*, 2*B*), I identified 16 that are known to be linked to immunity: 10 with direct immune function and 6 with auxiliary immune function (Fig. 2*C*, Table 1). Given that (a) MG has immunosuppressive effects on later phases of host immunity (i.e. after 1-2 weeks, see Introduction), (b) the microarray consisted of clones differentially expressed between infected and control birds 2-weeks post-inoculation, and (c) I examined transcriptional changes occurring in the later stage of the host immune response, the hypothesis of recently evolved resistance would predict population differences in susceptibility to immunosuppression and in the ability of birds to mount an immune response against MG. More precisely, it would predict that the evolution of resistance to MG would be associated with a post-infection up-regulation of genes involved in immunity or immune activation among finches from Alabama in 2007, and down-regulation of those genes in finches from Arizona in 2007.

In accordance with these predictions, of 11 immune-related genes differentially expressed between infected and control birds across both populations, 5 of 6 were down-regulated in Arizona and 5 of 5 were up-regulated in Alabama (Comparisons 1 & 2; *Fisher exact test*, $P = 0.015$). In addition, of the 10 genes with direct immune function and 6 with auxiliary immune function, 90% and 100%, respectively, displayed lower expression levels in infected birds from Arizona versus Alabama (Comparison 4) (one-sample *Binomial test* = 14.25, $P < 0.001$). Taken together, these results independently suggest that MG infection is associated with suppression of immunity in house finch hosts, and that birds from Alabama are better able to mount a more robust immune response to MG at the molecular level than birds from Arizona.

(3) Population changes in gene expression patterns

I used quantitative comparisons of 2007 expression patterns with those of a 2000 Alabama study to further test hypotheses regarding the emergence of resistance in eastern U.S. house finches (Fig. 3). Evidence against the MG-attenuation hypothesis as the only driver would again be supported if expression patterns between infected and control birds in 2007 in Arizona (Comparison 1) and Alabama (Comparison 2) were more similar to each other than to those of infected versus control birds in Alabama in 2000, since the latter used an earlier, potentially more virulent strain of MG. If long-term changes to individual body condition accounted for the emergence of resistance in eastern finches, then I would expect expression differences between infected and control birds to be a function of site of origin; differences should be more similar between years within Alabama, than between Alabama in 2000 and Arizona in 2007. By contrast, the hypothesis that resistance to MG involved genetic evolution in the host would be supported if expression changes between infected and controls in Alabama in 2000: (a) differed

from those in the same population in 2007 (Comparison 2) and resembled those in Arizona in 2007 (Comparison 1); and also (b) resembled the expression differences between infected birds from Alabama and Arizona in 2007 (Comparison 4). The first prediction arises because birds from Alabama in 2007 were expected to be resistant, whereas birds in Alabama in 2000 and in Arizona in 2007 were not. The second prediction arises because if birds from Alabama in 2007 have evolved resistance, infected finches from Alabama in 2000 and Arizona in 2007 should both display lower expression levels than control birds from Alabama in 2000 and infected birds from Alabama in 2007, respectively.

Overall, 14 genes were identified as being differentially expressed post-infection in both the 2000 and 2007 studies. Of these, 11 were down-regulated and 3 were up-regulated in 2000 (Wang et al. 2006). Whereas seven of the 14 genes showed expression changes in the same direction when comparing infected versus control birds from Alabama in 2000 and from Arizona in 2007 (Comparison 1), none of the gene expression changes were in the same direction when considering the Alabama population in 2007 (Comparison 2) (Fig. 3*A*). Thus responses to infection were more similar between eastern and western birds with little or no evolved resistance to MG, than among birds captured from the same sites but different stages of the epizootic (*Fisher exact test*, $P = 0.003$). In addition, 12 of 14 genes up- or down-regulated following infection in Alabama in 2000 showed a reversed direction of expression difference when comparing infected birds between Alabama and Arizona in 2007 (Comparison 4) (*one-sample binomial test* = 2.40, $P = 0.016$; Fig. 3*B*). In other words, infected birds from a population before the spread of resistance to MG (Alabama 2000) expressed genes at lower levels than did control birds from the same population. Similarly, infected birds from a population that had never experienced MG (Arizona 2007) expressed genes at lower levels than

infected birds from a population that had evolved resistance to MG (Alabama 2007). Taken together, these results rule out MG-attenuation or long-term differences in body condition as likely explanations for the emergence of resistance in eastern house finches, but fully support all predictions of the evolution of resistance hypothesis.

Discussion

Two weeks following an experimental infection conducted on wild-caught house finches in 2007, finches from the eastern U.S. (Alabama), with 12 years of exposure to the conjunctivitis-causing bacterium MG in the wild, harbored 33% less MG in their conjunctivae than finches from the western U.S. (Arizona), which had never experienced the disease. Furthermore, I detected distinct transcriptional responses between populations, both in terms of the number and direction of expression changes, in response to MG infection. In particular, infected birds from Arizona in 2007 showed significant down-regulation and reduced expression of immune-related genes compared to infected birds from Alabama in 2007. A comparison with a previous macroarray analysis of gene expression following similar experimental conditions (Wang et al. 2006) suggested that these transcriptional changes have evolved over the past 12 years in eastern finches and have hence accompanied the spread of resistance to MG.

Suggestions of rapid evolution based on phenotypic changes at the population level can often be attributed to phenotypic plasticity rather than adaptive changes in gene frequencies (West-Eberhard 2003, Badyaev 2008). Phenotypic plasticity could account for the emergence of resistance in populations of eastern house finches if individuals were able to acquire immunity during their lifetimes and pass it on to following generations (Boulinier & Staszewski 2008), or if environmental conditions in the recent past (i.e., after 2000) were more conducive to resistance

in the short- or long-term. My experimental setup in conjunction with measurements of phenotypes at the organismal and molecular level allowed me to distinguish between competing hypotheses that could potentially explain the emergence of resistance in eastern populations of house finches.

First, the lack of previous exposure to MG of the actual birds used in this study meant that differences in MG-load or gene expression changes following experimental infection could not be explained by acquired immunity. An alternative explanation however, is that infected mothers transmit antibodies against MG to developing offspring (Boulinier & Staszewski 2008), somehow conferring on them an early or more long-lasting advantage against MG. While such maternal effects could facilitate the spread of MG-resistance following an evolution of resistance, if maternal effects preceded the evolution of resistance, I would expect gene expression profiles at the two time points in Alabama to be more similar to each other than to Arizona in 2007. To the contrary, expression profiles in Alabama in 2000 were more similar to those in Arizona in 2007 than those in Alabama in 2007. Furthermore, evidence of immunosuppression in Arizona in 2007 and in Alabama in 2000 suggested that the transmission of maternal antibodies against MG is unlikely to have driven changes in disease dynamics in the wild (see below). Second, by maintaining all birds in identical conditions for three months prior to the onset of the experiment, I removed the possibility that the inter-population differences in MG-load and gene expression in 2007 could be caused by short-term condition-dependence. Nevertheless, the three-month acclimatization period would not necessarily entirely eliminate all differences in long-term condition indices arising from differing developmental conditions between sites (Metcalfe & Monaghan 2001). If ecological differences between Alabama and Arizona influence house finch immunity and gene expression, I would again expect expression

profiles within Alabama to be more similar. As indicated above, this was not the case, suggesting that inter-population differences in responses to MG-infection were independent of any differences in ecological conditions. Finally, the greater similarity in expression patterns between Arizona in 2007 and Alabama in 2000 indicated that attenuation of MG between 2000 and 2007 could not exclusively explain my results either. Taken together, the best supported explanation for my results is the evolution of host resistance by natural selection in eastern house finches over the 12-year period, from the fall 1995 to early 2007.

Examination of gene expression profiles further supported this conclusion. MG is well known for its complex immunomodulatory effects in poultry, which includes the suppression of important immune processes a week or two after MG inoculation (Ganapathy & Bradbury 2003, Mohammed et al. 2007). Consistent with the evolution of reduced susceptibility to immunomodulation, Alabama finches showed greater up-regulation (or increased expression when compared with infected Arizona finches) of immune-related genes, two weeks post-inoculation. Overall, all of the 16 immune-related genes identified were up-regulated in infected versus control birds from Alabama in 2007, whereas 83% were down-regulated in the same comparison in the Arizona population in 2007. In addition, all but one of the immune-related genes was expressed at higher levels in the infected birds from Alabama versus Arizona. One gene (hCG40889 or complement factor H) revealed an illuminating exception to this pattern. Unlike the other 15 immune-related genes which are all involved with counteracting infections (Table 1), complement factor H, which restricts the activation of the complement cascade to protect host cells and tissues, exhibited an expression direction opposite to that expected (de Cordoba & de Jorge 2008). Under MG-induced immunosuppression, an opposite expression pattern of complement factor H relative to other immune-related genes identified would be

expected. Thus, my results suggest that birds from Alabama in 2007 have evolved resistance to infections with MG and are able to counter MG-induced immunosuppression, an observation with important implications for the evolution of immunity in vertebrates (Schulenburg et al. 2009).

Evolution can arise through the emergence and subsequent selection of a novel mutation or through selection on existing (standing) variation in the population (Barrett & Schluter 2008). Experiments in *E. coli* reveal that adaptive mutations typically arise over hundreds or thousands of generations (Blount et al. 2008). The evidence that eastern house finches evolved resistance within 12 years suggests that genetic variability in resistance to MG existed at the time of outbreak. Selection by MG would then have produced a shift in allelic frequency reflected in the change in gene expression in the eastern U.S. finches over time and resulting in population-level changes in resistance to MG. In addition to helping us understand the evolvability of wild populations, my results may also help to predict the impact of an outbreak of MG that would reach Arizona. Given that the eastern U.S. finch population originated from western U.S. birds, it is reasonable to assume that standing variation for resistance is present in Arizona. Further, in my infection experiments, the MG load detected in the conjunctivae of two out of the 11 experimentally-infected Arizona birds ranked among those of the 10 Alabama birds (after excluding an individual from Alabama that showed no signs of resistance to MG, Fig. 1b). A simple extrapolation suggests that at least 2 in every 11 birds (~20%) would be likely to resist an MG outbreak in Arizona, which is close to the estimated 30% that survived the outbreak in Alabama (http://birds.audubon.org/historical-results), but this will depend on the virulence of MG (Hawley et al. 2010).

In conclusion, there are important implications from the observation that house finches exposed to MG have evolved resistance through changes in the expression of functionally-relevant genes within only 12 years. Few studies have shown that adaptive phenotypes can spread rapidly in wild vertebrate populations (Grant & Grant 2006, Linnen et al. 2009). In addition, I show that such a spread is associated with changes in functionally-relevant gene expression, an observation predicted by current evolutionary theory (West-Eberhard 2003), but previously confined to selection experiments in the laboratory (Ferea et al. 1999). Furthermore, while differences in gene expression have been hypothesized to indicate evolution in wild populations (Oleksiak et al. 2002, Abzhanov et al. 2006, Cheviron et al. 2008), the validity of this scenario requires evidence that observed differences have changed over time in response to an identified selective agent and also have functional significance (Fay & Wittkopp 2008). My study lends weight to the suggestion that differences in gene expression in the wild can reflect adaptive evolution (Oleksiak et al. 2002, Abzhanov et al. 2006, Cheviron et al. 2008) and indicates that population evolvability can be extremely rapid where sufficient standing variation exists.

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Table 1 Summary of the 52 genes found to be significantly differentially expressed in at least one of the four comparisons in the microarray and for which I identified a vertebrate homologue. I indicate the gene ontology category and function; many genes were implicated in several biological processes and I favored the processes associated with immune functioning or stress response. Gene ontology category and function were determined using Harvester (http://harvester.fzk.de/harvester/). Note that functions were mostly determined from studies on humans and mice, so although they are likely to be conserved, I cannot rule out that proteins have evolved to serve different roles in house finches. Genes with an identified auxiliary immune function are indicated as such in parentheses. Asterisks indicate genes that are also differentially expressed in the macroarray experiment (Wang et al. 2006); two new genes were identified with updated BLAST searches (Parathymosin, Eukaryotic translation initiation factor eIF4E).

Vertebrate homologue	Accession no.	*e*-value	Identity	Species	GO category	Gene ontology function	Expression change between infected and healthy
putative immunoglobulin J	DQ213324.1	0	709/804 (88%)	*Taeniopygia guttata*	Immune	Antigen binding	No
putative parathymosin variant 1	DQ214395.1	3E-180	517/594 (87%)	*Taeniopygia guttata*	Immune	May block prothymosin which confers resistance to opportunistic infections	No
putative MHC class II-associated invariant chain Ii	DQ215319.1	3E-131	462/550 (84%)	*Taeniopygia guttata*	Immune	Role in assembly of MHC class II	Yes – in AZ and AL
Predicted: immunoglobulin superfamily member 4A isoform a	XM_417901.2	3E-84	242/273 (88%)	*Gallus gallus*	Immune	Positive regulator of cytokine secretion	No
TCR beta chain	AF068228.1	8E-72	284/347 (81%)	*Anas platyrhynchos*	Immune	T cell recognition of foreign antigens	No
Predicted: lectin galactoside-binding soluble 2 protein	XM_002196008.1	4E-170	371/392 (94%)	*Taeniopygia guttata*	Immune	Regulator of cellular immune function	Yes- AZ only
PREDICTED: programmed death ligand 1	XM_424812.2	3.00E-45	200/245 (81%)	*Gallus gallus*	Immune	B cell differentiation, regulation of T cell activation	Yes- AZ only
Predicted: heat shock protein 90a	X07265.1	4E-130	310/337 (91%)	*Gallus gallus*	Stress	Molecular chaperone	Yes- AZ and AL
NADH dehydrogenase subunit 4	AY567938.1	0	639/773 (82%)	*Ciconia nigra*	Redox metabolism	Mitochondrial respiratory chain complex I	No
cytochrome b	AY495387.1	0	376/376 (100%)	*Carpodacus erythrinus*	Redox metabolism	Mitochondrial respiratory chain complex III	No
cytochrome c oxidase subunit I	EF484222.1	0	755/837 (90%)	*Mimus gundlachii*	Redox metabolism	Mitochondrial respiratory chain complex IV	Yes- AZ and AL
cytochrome c oxidase subunit II	EF484237.1	1E-56	153/167 (91%)	*Cinnyricinclus leucogaster*	Redox metabolism	Mitochondrial respiratory chain complex IV	No
cytochrome c oxidase subunit III	DQ385208.1	3.00E-64	208/242 (85%)	*Pedionomus torquatus*	Redox metabolism	Mitochondrial respiratory chain complex IV	No
cytochrome c oxidase subunit VIIa 2	DQ213599.2	0	444/459 (96%)	*Taeniopygia guttata*	Redox metabolism	Mitochondrial respiratory chain complex IV	Yes- AZ only
thioredoxin	EF192008.1	0	550/592 (92%)	*Taeniopygia guttata*	Redox metabolism	antioxidant activity, regulation of oxidative stress-induced signal transduction (Kondo et al 2006)	Yes- AZ only
Predicted: squalene epoxidase	XM_002187235.1	4E-175	628/664 (94%)	*Taeniopygia guttata*	Redox metabolism	Sterol biosynthesis, ROS production (Sanders et al 2002)	No
prosaposin variant 3	DQ214627.1	5E-59	168/187 (89%)	*Taeniopygia guttata*	Metabolism	Lipid metabolism and transport	Yes- AZ only
Predicted: RhoA GTPase	XM_002196158.1	0	546/558 (97%)	*Taeniopygia guttata*	Oxidative burst, signal transduction,	GTPase activity, subunit of NADPH oxidase,	Yes- AL only

Vertebrate homologue	Accession no.	*e*-value	Identity	Species	GO category	Gene ontology function	Expression change between infected and healthy
					immune	Rho protein signal transduction, motility of phagocytic cells	
Predicted: DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 3	XM_002190542.1	0	383/385 (99%)	*Taeniopygia guttata*	Translation	RNA helicase, Translation initiation	No
translation initiation factor eIF4E	DQ213184.1	0	818/831 (98%)	*Taeniopygia guttata*	Translation	regulation of translational initiation	Yes- AZ and AL
translation initiation factor EIF4G2 variant 1	NM_001099860.1	0	608/657 (92%)	*Gallus gallus*	Translation	regulation of translational initiation	Yes- AZ only
Predicted: ribosomal protein S24	XM_002195573.1	1E-117	254/264 (96%)	*Taeniopygia guttata*	Translation	Translation initiation	No
translation elongation factor 1 alpha 1	NM_204157.2	5E-116	244/250 (97%)	*Gallus gallus*	Translation	Translation elongation	No
ribosomal protein large P2	DQ213409.2	3E-92	202/209 (96%)	*Taeniopygia guttata*	Translation	Translation elongation	No
mediator complex subunit SOH1	EF191826.1	0	418/432 (96%)	*Taeniopygia guttata*	Transcription	RNA polymerase II transcription mediator activity, mediates activation of stress responsive kinases	No
nuclear ribonucleoprotein A/B (HNRNPAB)	NM_205328.4	1E-132	301/320 (94%)	*Gallus gallus*	Transcription	positive regulation of transcription	No
nucleic acid binding protein RY-1 variant 3	DQ216570.1	0	495/508 (97%)	*Taeniopygia guttata*	Nucleic acid binding	RNA splicing	No
Predicted: MAK-like kinase (ICK)	XM_002195712.1	0	478/488 (97%)	*Taeniopygia guttata*	Signal transduction	Protein phosphorylation	No
Predicted: sterile alpha motif and leucine zipper containing kinase AZK	XM_002198892.1	0	770/786 (97%)	*Taeniopygia guttata*	Signal transduction, pro-apoptosis, response to stress	Protein phosphorylation	Yes- AZ only
Predicted: Pleckstrin homology domain	XM_002189748.1	6E-166	346/359 (96%)	*Taeniopygia guttata*	Signal transduction	Binds inositol phosphates	Yes- AZ and AL
tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein (YWHAQ)	NM_001006415.1	1E-164	409/451 (90%)	*Gallus gallus*	Signal transduction	Protein scaffolding (Tzivion et al 2001)	Yes- AZ only
spermidine/spermine N1-acetyltransferase	EF192029.1|	2E-93	197/201 (98%)	*Taeniopygia guttata*	Polyamine Catabolism	Polyamine catabolism, response to inflammation	No
Predicted: Nedd4 family interacting protein 1	XM_002193395.1	0	390/399 (97%)	*Taeniopygia guttata*	Proteolysis	Ubiquitin mediated proteolysis	No
ubiquitin C	DQ216247.1	0	639/691 (92%)	*Taeniopygia guttata*	Proteolysis	Protein degradation (but see Boyer & Lemichez 2004)	No
putative hemoglobin	DQ216727.1	1E-	315/342	*Taeniopygia*	Oxygen		Yes- AZ

Vertebrate homologue	Accession no.	*e*-value	Identity	Species	GO category	Gene ontology function	Expression change between infected and healthy
alpha		132	(92%)	*guttata*	transport		only
cytoplasmic beta-actin	X00182.1	1E-48	144/159 (90%)	*Gallus gallus*	Cytoskeleton	Cell motility and structure	Yes- AZ only
Predicted: myosin regulatory light chain	XM_002186983.1	3E-150	312/322 (96%)	*Taeniopygia guttata*	Cytoskeleton	Regulator of myosin	No
destrin (DSTN)	NM_205528.1	5E-117	282/303 (93%)	*Gallus gallus*	Cytoskeleton	Actin-depolymerizing protein	Yes- AZ only
actin related protein 3 (ARP3)	AF498322.1	2E-86	304/361 (84%)	*Gallus gallus*	Cytoskeleton	actin polymerization	No
PREDICTED: lymphocyte cytosolic protein	XM_002198488.1	8.00E-125	266/276 (96%)	*Taeniopygia guttata*	Cytoskeleton	Actin binding protein in hemopoietic cell lineages	Yes- AZ and AL
Predicted: protein 4.1-G	XM_001232112.1	0	830/962 (86%)	*Gallus gallus*	Immune, cytoskeleton	T cell activation processes	Yes- AZ only
Predicted: Epidermal differentiation-specific protein	XM_002192313.1	7E-83	199/213 (93%)	*Taeniopygia guttata*	Cell differentiation		Yes- AZ only
Predicted: SEC61 gamma subunit	XM_002198448.1	2E-55	192/197 (97%)	*Taeniopygia guttata*	Transport	intracellular protein transmembrane transprt	Yes- AZ and AL

Fig. 1 Symptoms of *M. gallisepticum* infection in house finches and MG-loads. a) Naturally-infected (left) and healthy (right) wild house finches. b) Quantification of MG load in the conjunctiva of infected finches from Arizona and Alabama in 2007, two weeks post-infection. Figure shows raw values of MG load expressed as a ratio of host cell number; horizontal lines indicate mean values of raw data.

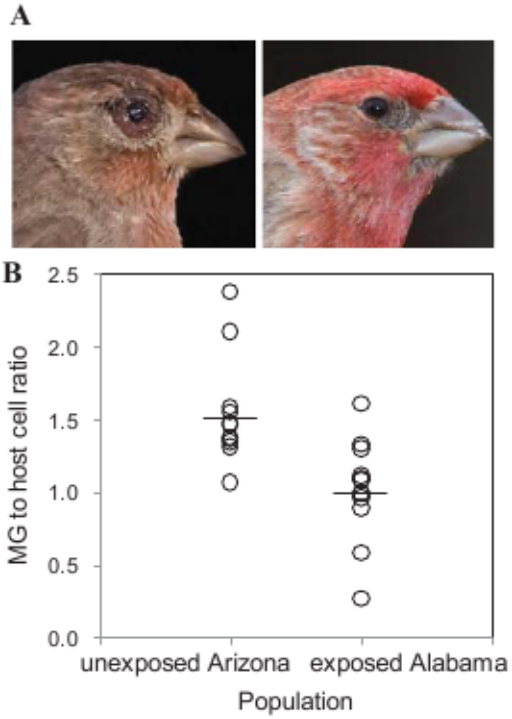


Fig. 2 Comparisons and patterns of splenic gene expression. a) Schematic of the four analytical comparisons made with gene expression data. b) Heat map of gene expression patterns in comparisons 1-4 (see Fig. 1b). Red and green indicate significantly higher and lower expression levels, respectively, with bright colors reflecting at least a 3-fold difference in magnitude and values in black indicating no difference. Comparisons in each of the 4 columns shown for 1st treatment/population *vs*. 2nd one outlined in Fig. 1b. The 52 genes included showed differential expression in at least 1 comparison (1-4) and were of known identity and function (Table 1 for full details). Asterisks indicate genes with direct and auxiliary immune functions. (***C***) Fold difference in expression levels of immune (*N*=10), immune-related (*N*=6) and stress (*N*=1) genes in Comparison 4. Genes shown were differentially expressed and known to have direct immune (I1-I10), indirect immune (R1-R3; Si1,P1,C1) or stress (St1) functions (Table 1). Negative values represent lower expression in infected birds from Arizona relative Alabama. Red (I1-I10) = immune genes: T-cell immunoglobulin and mucin dominant containing-4; MHC class II-associated invariant chain I1; lectin galactoside-binding soluble-2-protein; programmed death ligand 1; TCR beta chain; immunoglobulin J; neutrophil cytosolic factor-4; immunoglobulin superfamily member 4A isoform a; parathymosin; and complement factor-H. Yellow (R1-R3) = redox metabolism genes: thioredoxin; spermidine/spermine N1-acetyltransferase variant 1; and squalene epoxidase. Light (Si1), medium (P1) and dark (C1) blue = signal transduction (RhoA GTPase), proteolysis (ubiquitin C) and cytoskeleton (lymphocyte cytosolic protein) genes, respectively. Purple (St1) = stress gene (heat shock protein 90a). The stress gene was included because it was one of the few up-regulated in comparison 4, suggesting that birds from Arizona were more stressed by the infection.

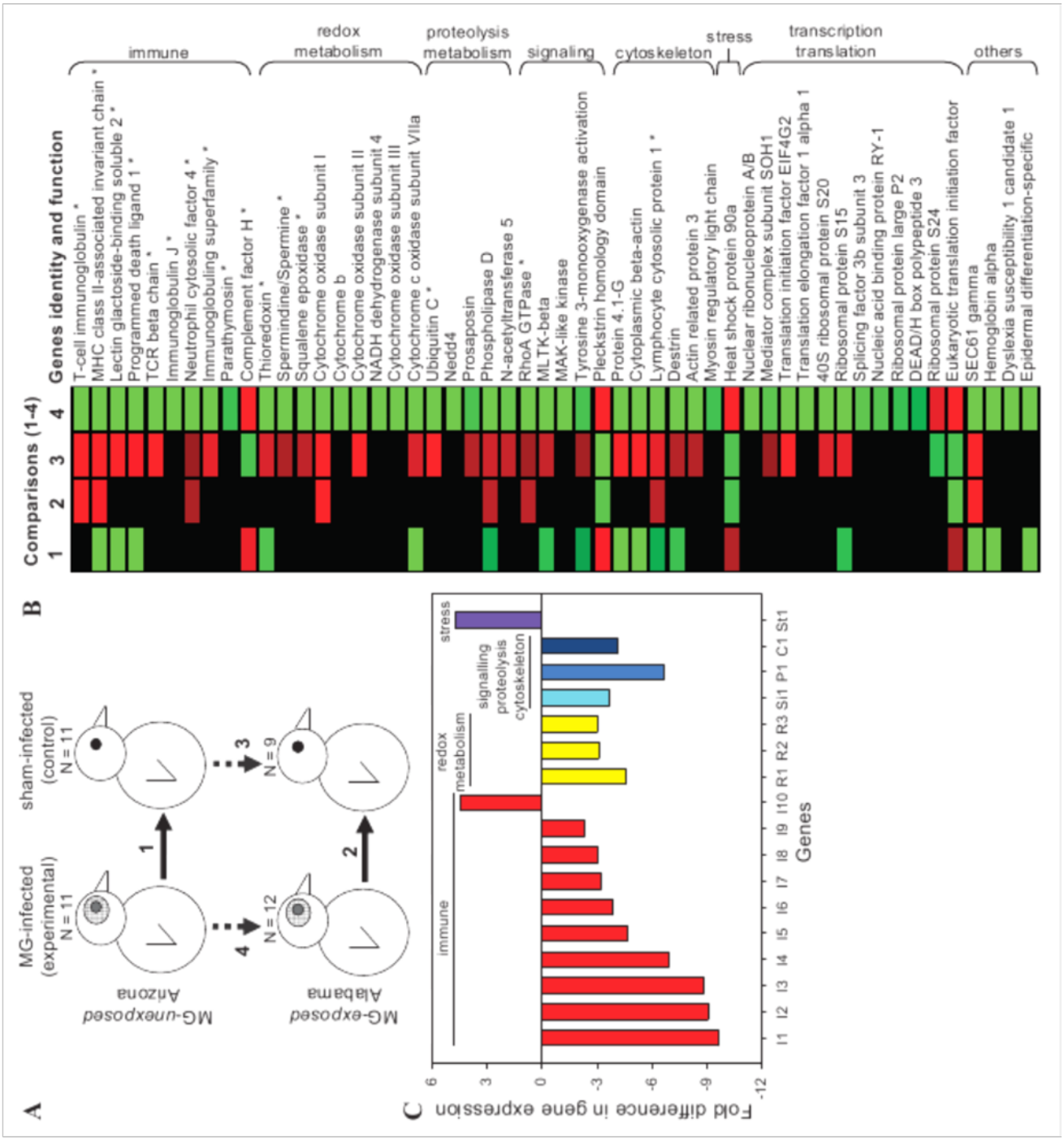
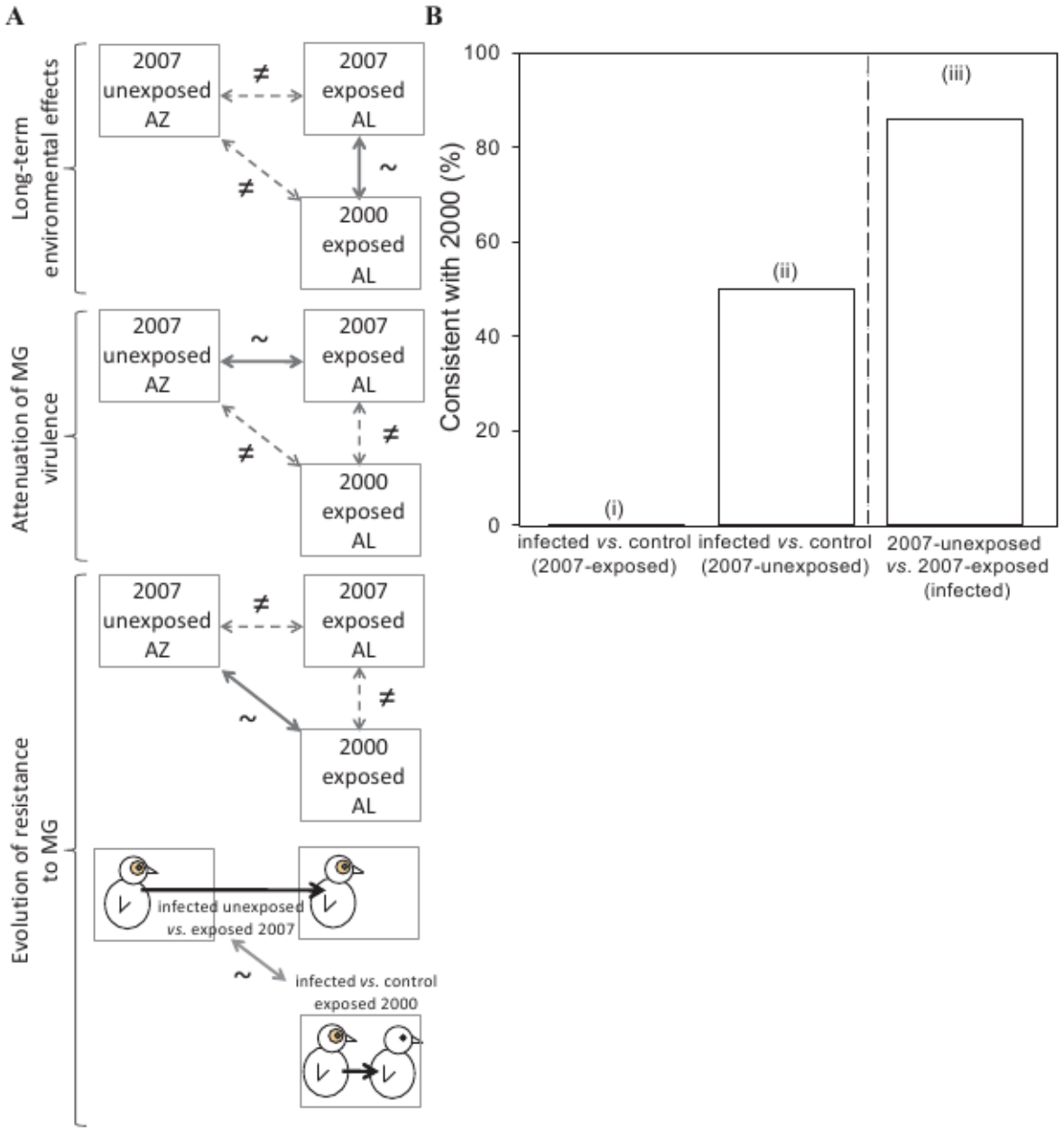

A
MG-infected (experimental) N = 11
sham-infected (control) N = 11
MG-unexposed Arizona
MG-exposed Alabama
1
2
3
4
N = 12
N = 9
B
Comparisons (1-4)
Genes identity and function
immune
redox metabolism
proteolysis metabolism
signaling
cytoskeleton
stress
transcription translation
others
T-cell immunoglobulin *
MHC class II-associated invariant chain *
Lectin glactoside-binding soluble 2 *
Programmed death ligand 1 *
TCR beta chain *
Immunoglobulin J *
Neutrophil cytosolic factor 4 *
Immunoglobuling superfamily *
Parathymosin *
Complement factor H *
Thioredoxin *
Spermindine/Spermine *
Squalene epoxidase *
Cytochrome oxidase subunit I
Cytochrome b
Cytochrome oxidase subunit II
NADH dehydrogenase subunit 4
Cytochrome oxidase subunit III
Cytochrome c oxidase subunit VIIa
Ubiquitin C *
Nedd4
Prosaposin
Phospholipase D
N-acetyltransferase 5
RhoA GTPase *
MLTK-beta
MAK-like kinase
Tyrosine 3-monooxygenase activation
Pleckstrin homology domain
Protein 4.1-G
Cytoplasmic beta-actin
Lymphocyte cytosolic protein 1 *
Destrin
Actin related protein 3
Myosin regulatory light chain
Heat shock protein 90a
Nuclear ribonucleoprotein A/B
Mediator complex subunit SOH1
Translation initiation factor EIF4G2
Translation elongation factor 1 alpha 1
40S ribosomal protein S20
Ribosomal protein S15
Splicing factor 3b subunit 3
Nucleic acid binding protein RY-1
Ribosomal protein large P2
DEAD/H box polypeptide 3
Ribosomal protein S24
Eukaryotic translation initiation factor
SEC61 gamma
Hemoglobin alpha
Dyslexia susceptibility 1 candidate 1
Epidermal differentiation-specific
C
Fold difference in gene expression
6
3
0
-3
-6
-9
-12
immune
redox metabolism
signalling
proteolysis
cytoskeleton
stress
I1 I2 I3 I4 I5 I6 I7 I8 I9 I10 R1 R2 R3 Si1 P1 C1 St1
Genes

Fig. 3 Comparisons of expression profiles at different stages of the epizootic. a) Hypotheses and predictions: long-term condition changes predicted that comparisons between infected and controls in Alabama in 2007 and in Alabama in 2000 would be more similar to each other than they would be to the one in Arizona in 2007; MG-attenuation predicted that Alabama in 2007 and Arizona in 2007 would be more similar to each other than they would be to Alabama in 2000; evolution of resistance to MG predicted that Arizona in 2007 and Alabama in 2000 would be more similar to each other than they would be to Alabama in 2007, and that the comparison infected Arizona *vs*. infected Alabama in 2007 would be similar to the comparison infected *vs*. controls in Alabama in 2000. Full arrows indicate greater similarity and dotted arrows greater dissimilarity. b) Of 14 expression differences found between infected and control birds from Alabama in 2000 (i.e., early in the epizootic): (i) none was common to those found in the same comparison in the same population later in the epizootic (in 2007), despite birds being captured from an identical location 7 years apart; (ii) 6 were qualitatively identical to those found in the same comparison but from the Arizona population in 2007, despite the two populations being isolated for at least 60 years; and (iii) 11 were qualitatively identical to differences found in infected birds between Arizona and Alabama in 2007.


A
B
Long-term environmental effects
Attenuation of MG virulence
Evolution of resistance to MG
2007 unexposed AZ
2007 exposed AL
2000 exposed AL
infected unexposed vs. exposed 2007
infected vs. control exposed 2000
Consistent with 2000 (%)
100
80
60
40
20
0
(i)
(ii)
(iii)
infected vs. control (2007-exposed)
infected vs. control (2007-unexposed)
2007-unexposed vs. 2007-exposed (infected)

II. PLUMAGE COLOR PREDICTS PATHOGEN-INDUCED GENE EXPRESSION IN A WILD BIRD

Abstract

The ornamental traits of animals can serve as reliable signals of the ability to cope with environmental challenges, but the mechanisms that link ornamentation and performance such as disease resistance have rarely been studied. In the house finch (*Carpodacus mexicanus*), plumage color is an important criterion in female choice, and plumage redness also predicts the ability of individuals to recover from a pathogen. To investigate the relationship between plumage coloration and the ability to cope with disease, I captured wild male house finches and infected them with the bacterium *Mycoplasma gallisepticum* (MG). I collected spleen tissue and used a cDNA microarray to compare the transcriptional profiles of infected finches with red and yellow plumage to uninfected controls. MG can suppress host immunity, and males with redder plumage showed evidence of resistance to immune suppression. Relative to controls, many more genes were differentially expressed in infected yellow birds compared to infected red birds, and most changes in gene expression in yellow males involved down-regulation in response to infection. Of particular relevance to immune responsiveness, MHC class II invariant chain, galectin-2, and T-cell receptor beta chain were all down-regulated in yellow but not red males. In the house finch, less down-regulation of genes related to immune responsiveness appears to be the mechanism by which redder males cope with mycoplasmosis better than less ornamented males.

Introduction

The brilliant plumage colors exhibited by many species of birds are among the most striking yet enigmatic traits in nature. Explaining the existence of ornamental traits like bright and bold coloration has posed a challenge to evolutionary biologists because such traits often appear to reduce survival. In recent decades, biologists have focused on the hypothesis that ornamental traits are signals of individual condition and that females gain either resources or good genes for offspring by choosing to mate with males with high expression of such traits (Andersson 1994). The honesty of such signals might be maintained by energetic trade-offs between ornament production and body maintenance such that only males with abundant resources can afford maximal ornament production (Halliday 1987,Wedekind & Folstad 1994, Westneat & Birkhead 1998). Alternatively, Hill (2011) recently suggested that ornamentation might signal performance if the cellular pathways needed for production of the ornament are linked to pathways essential to vital cellular processes.

One of the most fundamental aspects of individual performance that is hypothesized to be signaled through ornamentation is disease resistance. Hamilton and Zuk (1982) proposed that plumage coloration might correlate with specific alleles that confer immunity to specific pathogens such that females will gain good genes for offspring by choosing brightly colored males. On the other hand, plumage coloration may reveal fundamental aspects of functionality, such that brightly colored males have a better overall capacity to deal with environmental stressors, including parasites (Hill 2011). A fundamental prediction of both these hypotheses is that feather coloration should reflect the ability to resist and recover from pathogens. While many studies have reported correlations between parasite loads and color displays (Møller et al.

2000) and a few experimental studies have shown that infection by parasites inhibits maximum expression of colorful plumage (Brawner et al. 2000), only two studies have shown evidence that plumage color predicts the capacity of males to recover from infection (Lindström and Lundström 2000, Hill and Farmer 2005). The mechanisms by which ornamentation can predict the ability to resist or recover from a novel pathogen remain unknown.

Here I seek to better understand the mechanism by which plumage coloration of male house finches (*Carpodacus mexicanus*) predicts their ability to recover from a novel pathogen by measuring the relative expression of genes among infected and uninfected males. Plumage coloration in the house finch is a well-studied ornamental trait (Hill 2002). Male house finches have extensive carotenoid plumage coloration that varies from yellow to red. Carotenoid coloration is a condition-dependent trait in the House Finch whose expression is affected by conditions during molt including access to carotenoid pigments (Hill 1992, 1993), general nutrition (Hill 2000), and parasite load (Brawner et al. 2000, Hill et al. 2004). Female House Finches prefer to mate with red males (Hill 1990, 1991, 1994), and such males provide more food to their incubating mates and to young in the nest (Hill 1991, 2002; but see also Duckworth et al. 2003).

Like all wild birds, house finches are host to diverse pathogens. One important disease of some wild House Finch populations is *Mycoplasma gallisepticum* (MG), a bacterial pathogen first identified in eastern US house finch populations in 1994 (Ley et al. 1996). MG localizes to the respiratory tract and is characterized by conjunctival swelling and discharge (Ley et al. 1996). During molt, male House Finches infected with MG grow significantly less saturated and less red (i.e. more yellow) feathers than males not infected with MG (Brawner et al. 2000, Hill et al. 2004). Furthermore, red males successfully clear symptoms of MG infection faster than

yellow males (Hill and Farmer 2005). These studies suggest that feather color indicates a male's infection status during molt as well as his ability to fight infection.

In this study, I used a custom cDNA microarray to test for differences in gene expression between red and yellow male house finches following experimental infection with MG. I first asked whether there were significant transcriptional differences between infected and control birds related to ornamental plumage color. I then tested the hypothesis that red males show a more adaptive pattern of gene expression than do yellow males. My goal was not simply to describe the changes in gene expression associated with ornamental male color, but rather to test whether those changes might be the mechanism by which more highly ornamented males are better able to recover from MG infection.

I deduced a pattern of House Finch gene expression that is more adaptive following infection with MG based on a comparison of gene expression patterns of a western U.S. population of House Finches that at the time of sampling had never been exposed to MG, and an eastern U.S. population of House Finches that had been selected for MG resistance. Finches in the eastern population have been under strong selection from the detrimental effects of MG (Nolan et al. 1998, Dhondt et al. 2006) and should show a more adaptive pattern of response to infection with this pathogen than finches from unexposed populations. Consistent with this prediction, when exposed to MG in controlled infection experiments, eastern finches have a lower pathogen load than do western finches (Chapter 1). Moreover, eastern finches showed less down-regulation of immune or immune-associated genes compared to western finches, suggesting that eastern birds did not experience the immunosuppressive effects associated with MG infection (Chapter 1). For these reasons, I take the pattern of gene expression shown by eastern House Finches to be the more adaptive response to infection by MG. It is relatively rare for results of microarray studies

to be directly comparable due to many potential sources of variation (Suárez et al. 2009). In this case, however, both the current study and Chapter 1 examined the same length of infection (14 d) with the same MG isolate using the same microarray, as well as using the same methods for analysis of the microarrays. Thus, here I use the patterns of gene expression described in Chapter 1 to determine whether ornamental plumage color is associated with an adaptive response to MG infection. Specifically, if plumage color functions as a signal of disease resistance, I predict that (a) gene expression of red infected birds will resemble that of infected birds from the MG-exposed, eastern population (Alabama) when compared to controls, and that (b) gene expression of yellow infected birds will resemble that of infected birds from the MG-unexposed western population (Arizona) when compared to controls.

Materials and Methods

Establishment of birds at aviary

A flock of 12 male house finches was captured near Tempe, Arizona and shipped to Auburn University, Alabama after being held in cages less than 48 hr. For the duration of the experiment, birds were housed in pairs in wire-mesh cages (0.5 m^3) in temperature-controlled rooms (5'x7'x8') with large windows allowing for abundant exposure to natural light. Birds were provided with grit and fed sunflower seeds, brown and white millet, and water *ad libitum*, as well as apple slices and crushed eggshells on a weekly basis. All birds were maintained for three months prior to the start of experiment to allow them to acclimate to captivity and me to monitor for any diseases. Within one week of capture, I collected 15 rump feathers from each bird and taped them to a black card for color measurement (see below for details). I tested for exposure to

MG using a serum plate agglutination (SPA) assay (Luttrell et al. 1996) upon arrival in Auburn and at the end of the quarantine period. I also tested for the presence of MG DNA by PCR amplification of choanal swabs (Roberts et al. 2001). All birds used in this study tested negative for both tests.

Color analysis

I measured reflectance spectra of collected feathers across the avian visual range (300-700 nm) following standard procedures (Siefferman and Hill 2003, Perrier et al. 2002, Quesada and Senar 2006) to collect raw spectral data using an Ocean Optics S2000 spectrometer (Dunedin, FL). Following (Hill 2002, Hill and Farmer 2005), I used hue as the most relevant descriptor of male house finch color. I quantified hue as the wavelength of 50% reflectance (λR50) located at the midpoint between the maximum and minimum reflectance contained within the visible spectrum (Pryke et al. 2001).

Experimental groups

The feather hues of the 12 male House Finches captured for this experiment clustered into two groups: long wavelength hues (hereafter called *red;* n = 7, $\overline{x}$ = 610 nm; range = 599-619 nm), and short wavelength hues (hereafter called *yellow;* n = 5, $\overline{x}$ = 578 nm, range = 571-583 nm). Males in each color group were randomly divided into control and experimental treatment groups (red control n = 4; red infected n = 3; yellow control n = 2; yellow infected n = 3; Fig. 1). Control birds were sham inoculated by dropping 10 uL of sterile SP4 media into each eye of each bird. Birds in experimental infection groups were inoculated by dropping 10 uL of SP4 media to each eye. This media was from a stock culture containing approximately 1 x 10^4 to 1 x

10^6 color changing units/mL of an MG field isolate collected in Auburn, AL, January 2007 (BUA #243).

Control and infected birds were maintained under identical conditions but in separate containment rooms with separate entrances and self-contained air supplies (see Farmer et al. 2005 for detailed description of rooms). After exposure, birds were monitored daily for disease onset and progression of symptoms. Birds were bled 14 days post-infection to test for seroconversion and swabbed 3 and 14 days post-infection to confirm the presence of MG DNA in the choanal cleft (Roberts et al. 2001). All birds were euthanized 14 days post exposure (IACUC protocol #2007-1197). Spleens from all birds were removed immediately following death and stored in RNAlater at 4°C for 24 hours before being placed at -80°C for future characterization of gene expression.

Microarray construction

A detailed description of the house finch cDNA microarray slides is given in Chapter 1. Briefly, I constructed a cDNA microarray using differentially expressed and control cDNA clones from the Subtraction Suppression Hybridization (SSH) libraries described in (Wang et al. 2006). The microarray included approximately 1000 unique clones, including 220 clones found to be differentially expressed between healthy and infected House Finches in a previous study using macroarrays (Wang et al. 2006), as well as 694 randomly selected clones. I also printed five house finch housekeeping genes. These genes were generated by PCR amplification of cDNA extracted from House Finch spleens using degenerate primers. I quantified purified PCR products on a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE).

In addition, I printed the PCR products from the DNA amplification of 11 *Escherishia coli* housekeeping genes (Chapter 1) to serve as external spike-ins (van Bakel and Holstege 2004). I amplified these housekeeping genes as described in (Noller et al. 2003, Hommais et al. 2005). PCR products were purified, sequenced and quantified as described in Chapter 1.

Sample preparation and microarray hybridization

I extracted total RNA from approximately 17 mg of each individual's sonicated spleen tissue using Qiagen RNeasy miniprep spin columns, and digested genomic DNA using Turbo DNase, both according to manufacturers' protocols (Ambion, Austin, TX). I quantified the amount of total RNA using a Nanodrop spectrophotometer and determined the integrity of the RNA using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). RNA extracts were then stored at -80ºC until hybridization.

Because I was unable to extract sufficient quantities of RNA to hybridize each individual's spleen separately on a microarray slide, I pooled samples and hybridized them against a common reference. The common reference sample was made by pooling an aliquot of each of the individual samples included in the hybridization. After pooling samples, the final list of samples that I hybridized against the common reference was: infected yellow birds (n = 1 consisting of 3 pooled individuals), infected red birds (n = 1 consisting of 3 pooled individuals), and control birds (n = 2 consisting of 2 and 4 pooled individuals).

I made a calibration curve of hybridization efficiency by diluting the *E. coli* external PCR products at known concentrations (1:1, 1:2, 1:4, 1:50, and 1:100). Each hybridization was performed on one half-slide, thus, within-slide spatial variation in printing and hybridization was controlled for by comparing the signal from the 2 replicated grids hybridized. Between-slide

differences were controlled for by comparing the signals from the *E. coli* external spike-ins, the house finch housekeeping genes and the common reference on the different slides.

Pooled house finch RNA samples and *E. coli* external spike-in housekeeping genes were prepared for cDNA microarray hybridization as described in detail in Chapter 1. Following hybridization, microarray slides were scanned for visualization of fluorescent probes using an Axon 4000A microarray scanner (Molecular Devices, Sunnyvale, CA).

Microarray analysis and qRT-PCR validation

I scanned the microarray slides and flagged low quality spots using GenePix 4.0 software. Normalization included a background adjustment using the normexp method, a Lowess regression (two dimensional method) to smooth the data, and a scale normalization to control for variation between slides. Data smoothing was performed in R using the bioconductor/marray package. Background adjustment and scale normalization were performed in R using the limma package.

I determined gene expression differences between samples by fitting a generalized linear model to normalized $\log_2$ transformed signal ratios (sample versus reference), with treatment (healthy and infected with MG) and plumage color (red and yellow) as factors. P-values were adjusted for multiple testing using the False Discovery Rate (FDR) correction.
I identified clones that were significantly differentially expressed between the following groups: (1) infected red males vs. controls, and (2) infected yellow males vs. controls. I verified transcriptional patterns at 16 genes using multiplexed quantitative RT-PCR amplifications, as presented in Chapter 1. Technical details of the assay are provided in full in Chapter 2.

Sequencing and gene ontology analyses

I sequenced clones found to be significantly differentially expressed between groups as described previously (Chapter 1). Forward and reverse sequences generating a BLAST hit with an e-value $< 1\times10^{-20}$ with more than 100 nucleotides were categorized by their vertebrate homologues, while all other genes were considered to be unknown. Gene ontology category and function were determined using Harvester (http://harvester.fzk.de/harvester/).

Comparisons of gene expression between color phenotypes and populations

To assess the different profiles of gene expression between yellow and red males, I compared results to those in Chapter 1 in which I used the same cDNA microarray to identify patterns of gene expression following MG exposure in males from populations of house finches that were more adapted (Alabama) and less adapted (Arizona) to MG. Given the relative nature of microarray data, I compare the patterns of gene expression (i.e., up-regulated, down-regulated, no significant change) identified in comparisons of infected and control birds in Chapter 1 vs. comparisons of infected and control birds in this study (Fig. 2a).

Results

Experimental infections of animals

All experimentally infected birds tested positive for MG DNA in their choanal cleft when swabbed 3-d and 14-d after inoculation, and all were seropositive after two weeks. All control birds remained negative for MG DNA and were seronegative throughout the course of the experiment.

Comparisons of gene expression between infected color phenotypes and uninfected controls

I detected a total of 82 SSH cDNA clones that were significantly differentially expressed in the microarray analyses. All 82 were significantly differentially expressed between infected yellow birds and controls, while only seven of these 82 were significantly differentially expressed between infected red birds and controls. Sequencing of these clones revealed that some were redundant and corresponded to the same gene, so that I was able to identify a total of 18 homologous genes from 41 SSH clones (see Table 1 for a brief description of each gene identified). The remaining 41 clones either blasted to Zebra Finch (*Taenyopygia guttata*) mRNA of unidentified function (n = 18), or did not yield any hits (n = 23), and thus I consider their functions to be unknown. Of the 18 differentially expressed genes, four were found to be involved in immune processes, two were involved in stress response, two were related to redox metabolism and several others were involved in functions like the initiation or regulation of translation and cytoskeletal cellular remodeling (Fig. 3).

Males with red and yellow plumage showed significant differences in both the number and direction of expression changes following infection. Of the genes identified, all 18 showed post-infection expression changes in the yellow birds while only one was significantly different between infected red birds and controls. Thus, birds infected with the same MG-strain, but exhibiting differences in plumage coloration showed markedly different patterns of gene expression in response to infection by MG. Most changes in gene expression involved down-regulation in the infected yellow birds (78% or 14 out of 18 genes).

Comparisons of gene expression between color phenotypes and populations

Of the 18 genes identified as differentially expressed in this study, 9 were also identified in comparisons between infected birds and controls in Chapter 1 (Table 2, Fig. *2B*). When infected with MG, red males shared the same direction of change in gene expression in 56% (5/9) of these genes as did birds from Alabama, a population demonstrated to exhibit an adaptive pattern of gene expression. These same red males shared the same direction of change in gene expression in only 11% (1/9) of these genes as did birds from Arizona, a population demonstrated to exhibit a less adaptive pattern of gene expression in response to MG infection. On the other hand, when comparing the change in gene expression due to infection with MG of yellow males to those of the Alabama and Arizona population patterns, I found opposite relationships. Specifically, only 11% (1/9) exhibited the same pattern between yellow males and the adaptive pattern exhibited by birds from Alabama, while 100% (9/9) of the genes compared showed the less adaptive gene expression pattern of the Arizona population. Overall, red finches showed a pattern of gene expression more like that of birds from the more adapted Alabama population while yellow finches showed a pattern of gene expression more like that of birds from the less adapted Arizona population (Fisher's exact test, P=0.015).

Discussion

Following infection by a bacterial pathogen, male house finches with drab yellow plumage showed patterns of gene expression that were different than the patterns of gene expression shown by males with ornamental red feathers. Red males infected with MG showed only a few differences in gene expression when compared to controls, while infected yellow males showed many differences in gene expression, with a general pattern of down-regulation of genes related to immune responsiveness. Such down-regulation of genes related to immune responsiveness

suggests that the immune systems of yellow males were being suppressed as a result of MG infection (Ganapathy and Bradbury 2003, Mohammed et al. 2007, Chapter 1). Additionally, yellow males also showed down-regulation of genes with cellular protective functions (spermidine, heat shock protein 70b) and up-regulation of a stress-related gene (heat shock protein 90a), again suggesting a qualitatively poorer response to infection by the poorly ornamented yellow males.

Distinct patterns of gene expression by red and yellow house finches supports the hypothesis that plumage color signals the ability of males to resist MG infection, but this conclusion holds only if the patterns of gene expression shown by red males are functionally better than the patterns shown by yellow males. Mycoplasmas, including MG, are known to modulate their host's immune system to evade detection and MG infections have been associated with evidence of immunosuppression (Mühlradt 2002, Ganapathy and Bradbury 2003, Mohammed et al. 2007). Hence the pattern of down-regulation of genes related to immune response in yellow but not red males is consistent with red birds escaping the immuno-suppressive effects associated with MG. Furthermore, I used a second criterion by which to assess the adaptiveness of the patterns of gene expression measured in red and yellow finches. At the time of this study, some populations of house finches in North America had been subject to MG infection and had consequently been under strong selection to resist MG infection for approximately 12 years (e.g., Alabama finches), while other populations had never experienced MG infection (e.g., Arizona finches). The pattern of gene expression following MG infection among birds from exposed populations is expected to be associated with a greater efficiency at fighting MG than that of birds from the unexposed populations. This assumption was supported in Chapter 1 by the higher levels of infection following MG inoculation in birds from Arizona compared to the birds from Alabama.

Moreover, experimental evidence suggests that MG-exposed populations have evolved resistance to MG indicating that their pattern of gene expression in response to MG infection is more adaptive than that of the MG-unexposed population (Chapter 1). Thus, I tested the adaptiveness of the gene expression patterns of infected red and yellow males by comparing their gene expression relative to controls to patterns observed in Alabama and Arizona populations relative to controls. Overall, the gene expression of yellow males mirrored that of males from Arizona, the MG-unexposed population, following infection. Conversely, the pattern of gene expression by red males following infection was like that of birds from Alabama, the MG-exposed population. Thus, I interpret the pattern of gene expression of males with red plumage as being associated with greater resistance to MG infection than the pattern of gene expression by males with yellow plumage.

Based on these observations, plumage color in the house finch appears to serve as an indicator of an individual's general ability to combat infection by MG. MG is a novel pathogen for the house finches used in this study, so there was no opportunity for the evolution of the sort of co-evolutionary cycles proposed by Hamilton and Zuk (1982). Nevertheless, redder plumage was associated with a better response to pathogen infection. This more adaptive pattern of gene expression shown by redder male house finches is consistent with the hypothesis that red plumage coloration serves as an indicator of an individual's overall capacity to deal with stressful environments, including disease (Hill 2011).

While these observations are consistent with the good genes hypothesis, they do not constitute a rigorous test of the idea. I have no evidence that the patterns of gene expression that I observed in this study are heritable, and indeed previous research indicates that red males are generally healthier than yellow males (Hill 2002). The superior performance of red male house

finches is, however, also consistent with the hypothesis that ornamental traits are signals of the capacity of an individual to maintain vital cellular processes when faced with environmental challenges, and this capacity may or may not be genetically-based (Hill 2011). Under this hypothesis, I would expect red males that suffer less from MG infection to also perform better under other types of environmental challenges such as heat or cold stress, nutritional deprivation, or sustained physical challenge, but such sequential stress tests remain to be conducted in conjunction with tests of disease resistance.

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Table 1 Homologous genes of the House Finch cDNA clones found to be significantly differentially expressed between microarray hybridizations. I indicate the gene ontology category and function; many genes were implicated in several biological processes and when that occurred, I emphasize the processes associated with immune functioning or stress response.

Vertebrate homolog	Accession no.	*e*-value	Identity	Species	GO category	Gene ontology function	# clones	p-value
actin, gamma 2 propeptide	XM_002190324.1	1.00E-071	155/157 (90%)	*Taeniopygia guttata*	Cytoskeleton	Cell motility and structure	1	0.016
predicted: SEC61 gamma subunit	XM_002198448.1	6.00E-089	192/197 (97%)	*Taeniopygia guttata*	Transport	Intracellular protein transmembrane transport	1	0.016
myosin, light chain kinase	AF045285.1	1.00E-100	237/253 (93%)	*Gallus gallus*	Muscle contraction	Phosphorylates myosin II light chain	1	0.016
erythrocyte membrane protein band 4.1-like 2	XM_002192813.1	0	593/612 (96%)	*Taeniopygia guttata*	Immune, cytoskeleton	T cell activation processes	1	0.016
TCR beta chain	AF068228.1	1.00E-071	284/347 (81%)	*Anas platyrhynchos*	Immune	T cell recognition of foreign antigens	1	0.016
putative MHC class II-associated invariant chain Ii	DQ215319.1	1.00E-174	565/665 (84%)	*Taeniopygia guttata*	Immune	Role in assembly of MHC class II	17	0.016
predicted: pleckstrin homology domain	XM_002189748.1	1.00E-165	346/359 (96%)	*Taeniopygia guttata*	Signal transduction	Binds inositol phosphates	1	0.017
translation initiation factor EIF4G2 variant 1	XM_002197226.1	0	637/656 (97%)	*Taeniopygia guttata*	Translation	Regulation of translational initiation	2	0.017
predicted: lectin galactoside-binding soluble 2 protein	XM_002196008.1	4.00E-170	371/392 (94%)	*Taeniopygia guttata*	Immune	Regulator of cellular immune function	1	0.02
ubiquitin C	DQ216247.1	0	639/691 (92%)	*Taeniopygia guttata*	Proteolysis	Protein degradation	4	0.02
putative eukaryotic translation initiation factor 4E (eIF4E)	DQ213184.1	0	818/831 (98%)	*Taeniopygia guttata*	Translation	Regulation of translational initiation	1	0.029
ribosomal protein S15	DQ213656.1	5.00E-050	126/132 (95%)	*Taeniopygia guttata*	Translation	Translation initiation	1	0.029
similar to hCG40889, complement factor H	XM_002192303.1	8.00E-078	227/257 (88%)	*Taeniopygia guttata*	Immune	Activates complement cascade	1	0.032
Destrin	NM_205528.1	2.00E-143	523/631 (82%)	*Gallus gallus*	Cytoskeleton	Actin-depolymerizing protein	2	0.036
heat shock protein 70B	XM_002195700.1	6.00E-109	230/236 (97%)	*Taeniopygia guttata*	Stress	Cytoprotection	1	0.039
predicted: heat shock protein 90a	XM_002200572.1	9.00E-112	244/253 (96%)	*Taeniopygia guttata*	Stress	Molecular chaperone	3	0.039
prosaposin variant 3	DQ214627.1	3.00E-072	262/311 (84%)	*Taeniopygia guttata*	Metabolism	Lipid metabolism and transport	1	0.043
spermidine/spermine N1-acetyltransferase	XM_002195920.1 |	7.00E-093	197/201 (98%)	*Taeniopygia guttata*	Polyamine Catabolism	Polyamine catabolism, response o inflammation	1	0.046

Table 2 Directions of gene expression changes for infected birds from an MG-unexposed population and infected yellow birds when compared to controls, and infected birds from an MG-exposed population and infected red birds when compared to controls. These 9 genes were comparisons (between infected and control birds) and this study. Up-regulated genes or genes expressed at higher levels in the first group are indicated by "Up", down-regulated genes or genes expressed at lower levels in the first group are indicated by "Down," and non-significantly differentially expressed genes are indicated by "No change". Patterns that match predictions are presented in bold text.

Vertebrate homologue	"Arizona" Pattern	Yellow Pattern	"Alabama" Pattern	Red Pattern
MHC class II-associated invariant chain Ii	**Down**	**Down**	Up	No change
complement factor H	**Up**	**Up**	**Up**	**Up**
destrin	**Down**	**Down**	**No change**	**No change**
erythrocyte membrane protein band 4.1-G	**Down**	**Down**	**No change**	**No change**
heat shock protein 90a	**Up**	**Up**	Down	No change
lectin galactoside-binding soluble 2 protein	**Down**	**Down**	**No change**	**No change**
pleckstrin homology domain	**Up**	**Up**	Down	No change
sec61 gamma	**Down**	**Down**	Up	No change
T cell receptor beta chain	**Down**	**Down**	**No change**	**No change**

Fig. 1 Scatterplot of the rump hue (measured as λR50) of each male included in the microarray hybridizations. Longer wavelength hues represent those shifted towards the red portion of the spectrum, while short wavelength hues are shifted closer towards the yellow portion of the spectrum.

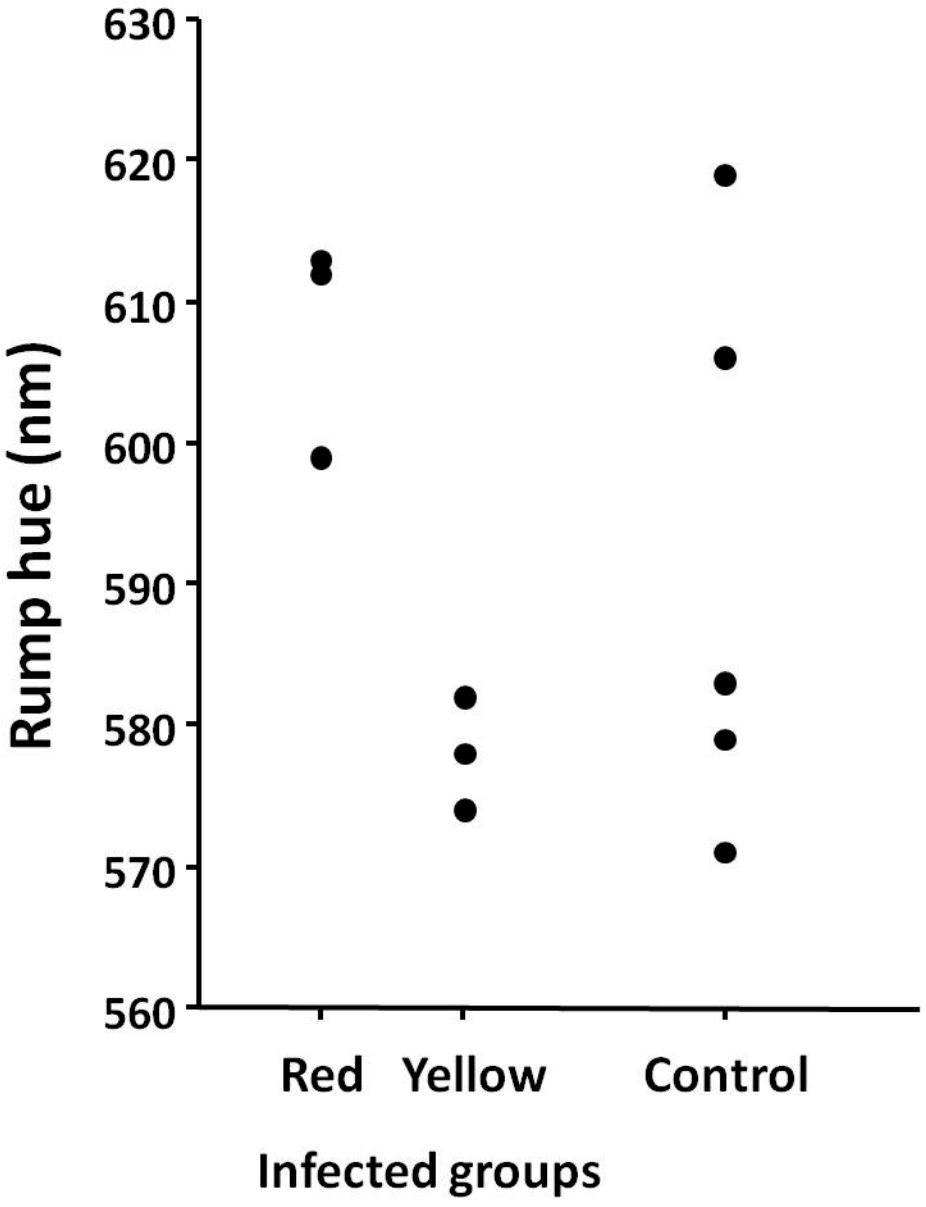
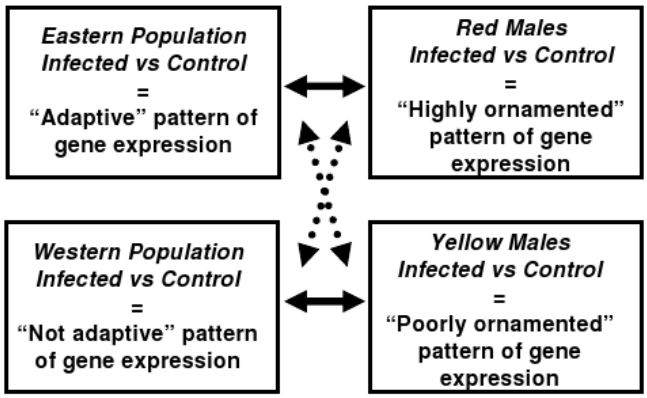


Fig. 2 Comparisons of expression profiles between infected birds and controls based on color and population history. a) Schematic illustrating qualitative comparisons made between results of Chapter 1 and this study. Each box represents a statistical comparison of gene expression between infected and control birds. The thick black bidirectional arrows represent comparisons that are predicted to reveal similar patterns (i.e., Red birds are predicted to exhibit an "Alabama" pattern, and Yellow birds are predicted to exhibit an "Arizona" pattern). The dotted arrows represent comparisons that are predicted to reveal dissimilar patterns (i.e., Red birds are predicted to not exhibit an "Arizona pattern", and Yellow birds are predicted to not exhibit an "Alabama pattern"). b) *Top panel:* All nine were common between yellow birds vs. controls (black bar) and one was common between infected red birds vs. controls (dotted bar) when compared to those found in the same comparison of infected birds vs. controls from Arizona, a population with no prior history of exposure. *Bottom panel:* Of nine expression differences between infected and control birds, one was common between yellow birds vs. controls (dotted bar) and five were common between infected red birds vs. controls (black bar) when compared to those found in the same comparison of infected birds vs. controls from Alabama, a population with a 12 year history of exposure to MG.

a)


Eastern Population Infected vs Control = "Adaptive" pattern of gene expression
Red Males Infected vs Control = "Highly ornamented" pattern of gene expression
Western Population Infected vs Control = "Not adaptive" pattern of gene expression
Yellow Males Infected vs Control = "Poorly ornamented" pattern of gene expression

b)

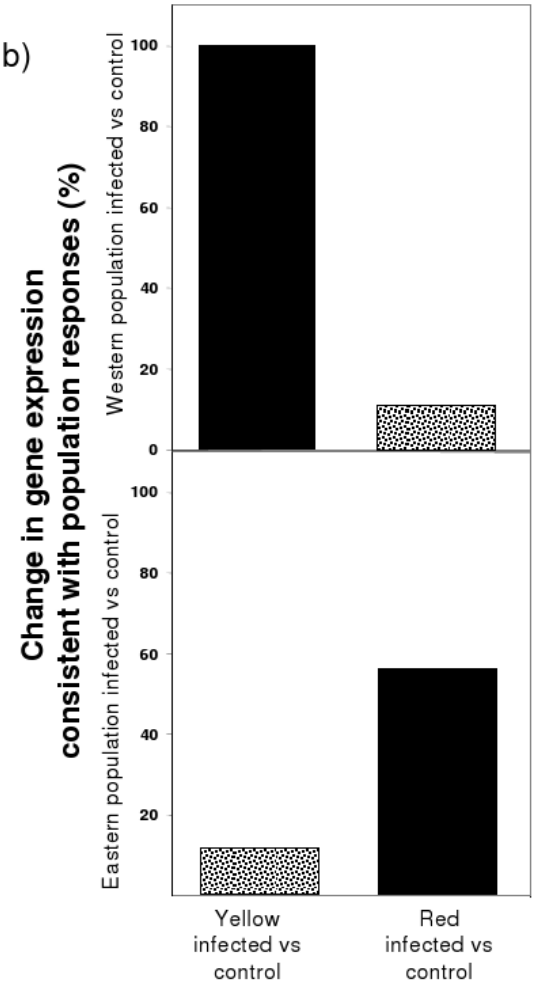

Change in gene expression consistent with population responses (%)
Western population infected vs control
Eastern population infected vs control
100
80
60
40
20
0
Yellow infected vs control
Red infected vs control

Fig. 3 Statistically significant changes in expression levels between infected yellow males relative to uninfected control birds for genes of known function. Bar colors represent general functional category determined using Harvester.

Immune
Translation
Muscle contraction
Proteolysis
REDOX/stress
Cytoskeleton
Protein transport
Signal transduction
2.0
0.0
-2.0
-4.0
-6.0
-8.0
Normalized Fold Change in Expression
mhc, class II invariant chain
lectin galactoside-binding soluble 2 protein
erythrocyte membrane protein band 4.1
TCR beta chain
spermidine/spermine N1-acetyl transferase 1
prosaposin variant 3
heat shock protein 70B
heat shock protein 90a
translation initiation factor 4G2
ribosomal protein S15
translation initiation factor 4E
actin, gamma 2 propeptide
destrin (actin depolymerizing factor)
myosin, light chain kinase
SEC61, gamma subunit
ubiquitin C
pleckstrin homology domain interacting protein

III. PRIMER DESIGN AND TRANSCRIPT QUANTIFICATION OF A HIGHLY MULTIPLEXED RT-PCR FOR A NON-MODEL AVIAN SPECIES

Abstract

Multiplexed qRT-PCR assays are currently lacking for nearly all species without genome or transcriptome resources. Here I present a strategy for primer design of highly multiplexed qRT-PCR assays, evaluate Beckman Coulter's Quant Tool gene expression quantification software, and provide details of my assay for the North American songbird *Carpodacus mexicanus* (house finch), for which only small sections of genome sequence are available. I combined Beckman Coulter's eXpress Designer module for creating custom multiplex primers with the free, online program Amplify 3 to design and evaluate primers computationally before testing them empirically. I also generated a standard curve for each gene included in the final multiplex. I compared models using cubic and quadratic polynomial estimators that did and did not force the intercept through zero. Ultimately, I used the sequences available for 316 clones differentially expressed in cDNA macroarray and microarray comparisons, and from these sequences I was able to generate a set of transcript-specific primers for use with the GeXP analyzer for 20 house finch genes.

Introduction

The expanding use of large scale, high-throughput genome and transcriptome datasets generated from microarrays and pyrosequencing projects has renewed interest in examining variation in functionally relevant and adaptive markers in the context of individual and population fitness (Vasemagi & Primmer 2005, Hoffman & Willi 2008, Shiu & Borevitz 2008, Ungerer et al. 2008,

Piertney & Webster 2010). Studies of gene expression expand our ability to identify and understand the function of genes, but traditional platforms for assaying transcript abundance are not always ideal for studies of natural populations. Specifically, microarrays and real time-PCR platforms are useful for transcriptome-scale and single-gene studies respectively, but the cost and complexity of microarrays limit their use by many researchers while extensive lists of candidate genes often cannot feasibly be examined using real time-PCR. Employing multiplexed RT-PCR platforms to simultaneously measure the expression of multiple genes can significantly reduce time and cost as well as improve measures of intra-sample variation. Currently, multiplexed RT-PCR assays have been primarily implemented in biomedical research, including neuropathological disease diagnosis, cancer-biomarker signatures, and viral infection identification (Therianos et al. 2004, Chen et al. 2007, Rai et al. 2009, and Nagel et al. 2009). The lack of ecological and evolutionary studies utilizing these assays is likely due in large part to the difficulty involved in designing primers that will not amplify off-target transcripts, which is especially difficult when working with a species lacking genome or transcriptome sequences. Furthermore, each gene in a multiplex typically requires its own distinct fluorescent probe so that it can be distinguished from other genes (Brisson *et al*. 2004). The need for individual labeling typically limits multiplexed platforms to between 4 and 6 genes. The cost of multiple probes can increase the cost of development of the assay to the extent that it makes multiplexing impractical.

Beckman Coulter offers its GeXP gene expression platform for examining up to 35 genes in a single multiplexed reaction. Using the Beckman CEQ8000 DNA series gel capillary electrophoresis sequencer, genes are differentiated based on a unique amplicon size rather than

the fluorescence wavelength of the probe. Thus, only a single dye is used for every gene of interest by virtue of a complementary universal sequence tag attached to each forward primer.

Recently, Rai et al. (2008) demonstrated an analytical validation of the GeXP method using a standard curve to assess inter- and intra-assay precision along a range of concentrations. More recently, Beckman Coulter released a normalization macro it calls 'Quant Tool', which is available for free download to CEQ8000 users. This tool incorporates sample expression data into the standard curve values, which allows for the relative quantification of transcripts of each gene included in the multiplex reaction according to its own standard curve. Quant Tool calculates the best fit line for each gene using a third order polynomial function with a y-intercept equal to zero.

This paper (1) presents my primer design strategy, which reduces the time and cost of primer design and optimization, (2) evaluates Beckman Coulter's Quant Tool gene expression quantification software as well as other possible polynomial estimators, and (3) provides details of the multiplexed qRT-PCR assay I designed according to these protocols for the North American songbird *Carpodacus mexicanus* (house finch), which has only a tiny amount of genome sequence currently available. I chose candidate genes for the *C. mexicanus* assay based on their association with an adaptive response to infection with the bacterial pathogen, *Mycoplasma gallisepticum* (Chapters 1 & 2), hereafter referred to as MG.

Materials and Methods

Transcript selection and primer design

I initially selected 30 House Finch genes of interest as well as three housekeeping genes to attempt to include in a single, multiplexed reverse transcriptase-PCR based on differential expression in a microarray study (Chapters 1 & 2) as well as interesting GO (Gene Ontology) functions (http://www.geneontology.org/). According to Beckman Coulter standard protocols, I also included a pair of primers designed to amplify an external RNA transcript spiked into each reaction.

I generated 'first pass' multiplex primers using the Primer Design module of the GeXP eXpress Profiler software (Beckman Coulter, Fullerton, CA). Each forward and reverse primer included a 5' end containing a universal priming sequence and a 3' end containing a transcript-specific sequence. Primer pairs were designed to yield RT-PCR products at least four nucleotides apart in length within a range of 100-400 nt, as well as having similar GC content and similar melting temperatures.

I imported primer and available gene sequence information from this 'first pass' multiplex into Amplify 3 (http://engels.genetics.wisc.edu/amplify/), which is a free online PCR simulation program for Mac OSX. In order to adapt Amplify 3 for RT-PCR primers, I created two sequence files in alternate orientations for each gene. I prepared two separate lists of primers: one containing all of the reverse primers and another containing both reverse and forward primers. I then simultaneously compared the entire set of primers to be multiplexed against each individual gene sequence, comparing only reverse primers against the 'RNA' sequence, and both reverse and forward primers against its reverse complement. Only fragments predicted to overlap with the size range of interest (i.e., 100-400 nt) were used in comparisons. I evaluated fragment sizes as well as the quality estimates given by Amplify 3, termed 'primability' and 'stability'. I then considered the entire pool of fragments and excluded target primers from the multiplex if they

amplified any undesigned fragment that overlapped the size (± 2 nucleotides) of a designed fragment. Amplification was expected when each primer in the pair had a primability value ≥ 80 and a stability value ≥ 50.

I needed to design new primers for Amplify 3 testing for those genes with primers that were excluded in the process described above. I subjected the genes to the Primer Design module of the eXpress Designer software. I used the Primer Design module to redesign primers for individual genes using default parameters, and tested these newly designed primers along with the rest of the primers in the multiplex using Amplify 3 as described above. I repeated this process until I identified primer pairs for each gene that met my criteria from a bioinformatic standpoint.

Animals & RNA

I collected total RNA from spleen tissue of male house finches that were and were not experimentally infected with MG. Specific details of capture, housing, care, and experimental manipulations of house finches are described in Chapters 1 & 2. Briefly, wild caught birds originating from two distinct populations were inoculated in each eye with 10uL of sterile SP4 media to each eye, or with 10uL of a stock culture containing approximately 1×10^4 to 1×10^6 color changing units/mL of an MG field isolate collected in Auburn, AL, January 2007 (BUA #243). Control birds were euthanized 14 days after sham-inoculation, while infected birds were euthanized either 3 or 14 days post exposure (IACUC protocol #2007-1197). I immediately removed the spleens from all euthanized birds and stored them in RNAlater at 4°C for 24 hours before placing them at -80°C for future characterization of gene expression.

I extracted total RNA from approximately 17 mg of each individual's homogenized spleen tissue using Qiagen RNeasy miniprep spin columns, followed by digestion of genomic DNA according to the manufacturers' protocols (Turbo DNase, Ambion). I determined the quantity of purified total RNA using a Nanodrop spectrophotometer and determined RNA integrity on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). All RNA extracts were stored at -80ºC until further processing.

Primer pool testing and multiplex optimization

I followed Beckman Coulter protocols for testing primer combinations and attenuating primer concentrations at the bench (www.beckmancoulter.com/literature/Bioresearch/A29143AC.pdf). Briefly, I tested all of the following possible combinations of primers with each sample pool in duplicate: (1) single reverse primer-single forward primer, (2) single reverse primer-multiplex of all forward primers, (3) multiplex of all reverse primers-single forward primer, and (4) multiplex of all reverse primers-multiplex of all forward primers. I then compared these results to those predicted by Amplify 3. Whenever (1), (2), or (3) produced UnDesigned Peaks (UDPs) of significant size (>2000 rpu) within 2 bp of any expected fragment (see Tables 1 and 2), I either removed the problematic primers from the multiplex, or, whenever possible, extended the primer by inserting a 1-2 nt spacer between its gene specific and universal tag portions to shift the amplified size away from the UDP.

Multiplexed qRT-PCR conditions

I generated a standard curve for all genes in the multiplex using a two-fold series of dilutions (250.0, 125, 62.5, 31.3, 15.6, 7.8, 3.9, 2.0 and 1.0 ng) of a reference pool of total RNA. Each

concentration was run in quadruplicate. The house finch reference pool comprised equal parts of RNA from each of two control and four treatment groups of birds (see Table 1 for details). I used Beckman Coulter protocols for each reverse transcription reaction using GeXP Start Kit reagents unless otherwise noted: 4.0 uL of RT buffer (5x), 2.0 uL of a pool of attenuated reverse primers (10x; Operon, Huntsville, AL), 1.0 uL of reverse transcriptase, 3.0 uL of DNase-free water, 5.0 uL of 0.625 ng/uL external spike-in control RNA (KAN^R) and 5.0 uL of 5 ng/uL sample mRNA. The concentration of each reverse primer varied from 0.01 to 5 umol/L to adjust the signal of each gene to within the dynamic range of the CEQ8000 fluorescence detector. Thermal reaction parameters for the RT reaction were 48°C for 1 min, 42°C for 60 min and 95°C for 5 min. Each PCR reaction consisted of 4.0 uL of $MgCl_2$ (ABgene, Rockford, IL), 2.0 uL of a 10x pool of forward primers (Operon) all at 2 umol/L concentration, 0.7 uL of Taq polymerase (ABgene) and 9.3 uL of cDNA from the RT reaction. Cycling parameters were 95°C for 10 min, followed by 35 cycles for 94°C for 30 sec, 55°C for 30 sec and 50°C for 1 min.

Quantification models

I generated a standard curve for each gene using the Quant Tool estimator, which produces a cubic polynomial estimator with a y-intercept forced through zero and uses the mean value measured at each concentration in generating the coefficients. I also examined cubic polynomial equations, which included each individual replicate in generation of the coefficients. The cubic lines included all replicates that were and were not forced through a zero intercept. I also generated a quadratic equation to examine the appropriateness of the cubic polynomial as the correct shape across the range of the instrument.

I was unable to rank the Quant Tool line estimator in comparison with my best-fit lines due to unequal sample sizes, so I limited my analysis to the three best-fit lines generated from replicates for each gene. I ranked and compared the three models separately for each gene using Akaike's Information Criterion (AIC, Akaike 1974) corrected for small sample size (Burnham and Anderson 2002). Model ranking was done using R. I then used a Chi-Square test to determine if one model consistently received the most model weight.

Validation of multiplexed qRT-PCRs

Chapter 1 gives details of construction and hybridization of a house finch cDNA microarray construction and hybridization. Cross-platform validation was performed using both a Spearman's rank correlation to test whether the magnitude of differential expression in the microarray was consistent with that in the multiplexed qRT-PCR and a one-sampling t-test framework to examine whether the direction of expression in the microarray and in the qRT-PCR was consistent. *P* values represent one-tailed estimates.

Results

Utilizing only the partial sequences available for 30 genes identified as differentially expressed in a cDNA microarray comparison, I was able to generate a set of transcript-specific primers for use with the GeXP analyzer for 20 House Finch genes (Table 2; Fig. 1).

I excluded between seven and nine replicates ($\overline{x}$ = 7.2, SD = 0.6) from the standard curve of each gene due to limits in the detection capabilities of the CEQ8000. Therefore, although each concentration of each gene should have included four replicates, the number of replicates was most commonly three, and ranged from one to four. Table 3 gives equations for best-fit lines

and r^2 values generated by the Quant Tool for each gene in the multiplex as well as those for the best model according to AIC ranks. In general, the model that included the cubic polynomial of the known concentration with the intercept forced through the origin received the most model weight (X^2= 24.72, df=2, p < 0.0001). The cubic polynomial model without the intercept forced through the origin, however, was the best model for three genes (Table 4), suggesting that the intercept may best be chosen quantitatively for some genes.

The house finch multiplexed qRT-PCR assay was validated using two different analytical approaches to compare it to a cDNA microarray study (data also given in Chapter 1). A correlational approach showed a magnitude of differential expression in the microarray consistent with that in the qRT-PCR (Spearman's rank correlation; R_S=0.40, N=44, P=0.002). A one-sampling t-test framework showed a similar direction of expression in the microarray and in the qRT-PCR: control birds from two distinct populations (T_{10}=2.26, P=0.024) and infected birds from two distinct populations (T_{15}=3.33, P=0.002).

Discussion

Multiplexed qRT-PCR assays are currently lacking for nearly all species with little available sequence because of the difficulty of designing primers that will work appropriately in a multiplex for such species. I present a protocol for primer design and analysis of multiplexed quantitative RT-PCR assays that is useful for a species with little available sequence. By combining Beckman Coulter's eXpress Profiler primer design software with Amplify 3, I was able to successfully select primers for 18 out of 30 genes of interest and 2 out of 3 housekeeping genes for inclusion in a single house finch multiplexed qRT-PCR gene expression assay. In

particular, I used Amplify 3 to test primers designed by eXpress Profiler and results were consistent in vitro and bioinformatically.

Beckman Coulter's eXpress Profiler suite of applications includes an option for designing primers for use in custom multiplexes (eXpress Designer). These programs provide what Beckman Coulter refers to as a "first pass" multiplex. In this module, users select which gene sequences or accession numbers to include in the multiplex and can also define the minimum desired difference in size between amplified fragments and the range of fragment sizes desired. This latter feature is limited by the maximum size standard used in the reaction, which is 400 bp. The eXpress Designer will immediately return one pair of primers for each gene selected for inclusion according to default parameters for melting temperature and primer length. Primer pairs from this "first pass," however, are designed by the software without regard for 1) sequences of the other genes of interest, 2) primers designed for use in the same multiplex, or 3) additional genome sequence information that may be available on public databases.

For researchers studying species with little genome sequence information publicly available, a post hoc database search can be frustrating and potentially unproductive. On the other hand, when a genome is available, results of searches can yield overwhelming amounts of information. Not performing a bioinformatic check, however, is also not advisable due to the cost required to empirically test and optimize each new primer pair to be added to a multiplex. Software such as Amplify 3 for Mac OSX provides a free and efficient method for evaluating multiple primers simultaneously against DNA sequences in a PCR simulated reaction. Amplify 3 software currently only simulates PCRs, but it is easily adapted for use in testing RT-PCR primers.

Amplify 3 allowed me to do two things that would otherwise not have been possible: 1) to compare the entire set of pooled primers simultaneously against a gene sequence, and 2) to

compare the primer plex against other sequences contained in my cDNA library which are currently not available through NCBI. The ability to compare a multiplexed primer pool against each gene of interest dramatically increased my ability to detect instances of non-paired primers amplifying undesigned fragments, while limiting the scope of the search to a manageable set of comparisons.

In the case of the house finch, I began with extremely limited sequence information (i.e., 316 clones ranging in length from 91 to 1,011 bp which were sequenced from a SSH cDNA library as part of macroarray (Wang et al. 2006) and microarray studies (Chapters 1 & 2)), and thus my ability to redesign primers was limited for many genes of interest. Of the genes included in the final multiplex, clone lengths ranged from 216 to 806 bp. Thus, while I began with some sequence information pertaining to each gene of interest, approximately one-third were ultimately excluded either as a result of predicted UDPs or after empirical testing at the bench. Having incomplete gene sequences for candidate genes limited the ability of Amplify 3 to detect potential UDPs as well as my ability to redesign primers. Many of my sequences were so short that redesigning primers would have shifted the fragment size below the size standard included in the reactions. In such situations, it would be useful to have an extended size standard available. Of course, many of the transcripts expressed in my samples were unknown and so could not be tested with Amplify 3 and are likely responsible for any UDPs I eventually detected. By combining bioinformatic and simulated reactions with laboratory generated data, however, I was still able to create primers for a highly multiplexed gene expression assay in a comparatively short period of time and at a reduced cost.

One of the advantages of using multiplexed RT-PCRs rather than microarrays is the relatively tiny amount of RNA required for this type of assay. Generation of a standard curve,

however, requires microgram quantities of RNA rather than the nanogram quantities required for running samples. The ability to generate expression data on up to 35 genes at once, typically requiring only 25 ng of total RNA for each reaction, lends itself to the types of investigations of individual variation that are of interest to ecologists and evolutionary biologists. However, if one is interested in establishing a quantitative scale using a standard curve, it is essential to consider the amount of sample that is required for generation of such a curve when planning experiments.

Finally, when estimating transcript abundance from a standard curve, I recommend considering whether or not to force the curve through zero, as is the default option for Beckman's Quant Tool software. Given that the line function of the standard curve is a 3rd order polynomial, forcing the fit through zero can have large effects on the values at the lower end of the range. In the current study of gene expression in house finch spleen, I used a simple estimator using R (www.r-project.org) that allows the user to determine whether to force the y-intercept through zero and that also allows for each replicate of the curve to be included as a data point in the fit of the predicted line. Including replicates in the fit of the line increases the degrees of freedom of the model and weights each concentration along the line according to the number of replicates. This is important because, especially at the high and low ends of the dynamic range of the instrument, one or more of the replicates may not be interpretable by the CEQ8000. When this occurs some concentrations along the curve will have more replicates than others, and thus, some points can be estimated with greater certainty than others. Unequal variance is a violation of the assumptions of linear regression and should thus be avoided. Further, using mean values in regression artificially reduces the variation in the data and inflates r^2-values. In many cases it may be possible to simply rerun the standard curve until each point is represented in quadruplicate. When samples are limited in quantity, as was the case of in this

study, multiple runs may not be an option. The Quant Tool estimator completely ignores this possibility, and I argue that the r^2-values produced by the Quant Tool estimator are invalid. Thus, the estimator presented here results in truer representations of the data generated by the GeXP system.

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Table 1 Contents of each reference pool of total RNA used to generate standard curves.

House Finch Groups
Eastern, Control
Western, Control
Eastern, 72-hr infection
Western, 72-hr infection
Eastern, 2-wk infection
Western, 2-wk infection

Table 2 Oligonucleotide primer sequences and expected PCR product sizes for House Finch multiplexed RT-PCR.

Gene symbol	GenBank acc. no.	Forward primer w/o universal tag (5'-3')	Reverse primer w/o universal tag (5'-3')	Product size
MAP	GW346167	TCTGGCCCAAAACTTCCATA	CCACATTCCCTTCTTCTTCTG	139
TXN	GW346164	GCAGCCTGGTTGAATTTGAG	AAAGGGCTTGATCATTTTGC	145
Ig4A	GW346137	CTCGTAGTGCAGTGTTACCGATGT	GGCCGAAATTTTGCAATCTA	149
DSTN	GW346131	CAGATGCTTCTGAGGCTTTTA	AGACTGGGGCTGAAAATACCA	152
LCP	DR782758	GGCATAGACTCTTGCTCCGA	TCAACCAGGGTCCATCAAGT	158
PPN	DR782822	GCTCTTGATGTGAAGGGTCC	CTCCTTCCCAAAGGTCTCCT	181
RHOA	GW346157	CGCCAAGCTCAGAATTAACC	CTCAGGAGATTGGCAGAAGC	188
SEC61	GW346160	TGGGATATGGATCAACCTGA	ATGCAGTTTGTGGAACCCAG	206
TCR	GW346163	AAACTGGCAACACACTCGAA	ACCCTGACCACCTGACTCTG	213
HSP90	DR782747	TGGAGACCTTTGCCTTTCAG	CAGCTCTTTCCCAGAATCCA	218
ICK	GW346139	AAACCATGCCAATGTGGTAAA	CCTGTGAAAGAACCCATGCT	240
TIF	DR782722	TTGGTTCAGCTCCCAATCTC	AAACAGGGATGCTGTTACGC	248
NADH4	DR782776	GCTGTGGGTTCGTTCGTAGT	AGGAGCAATCATAACCAGCG	255
UBC7	GW346169	CTGAGAGGTGGGATGCAGAT	ACAAGGTGCAGGGTGGATT	258
NABP	GW346152	ATAGCTTCAGACAGGGCGAA	TCCCAGCTAGCACTTAGGGA	263
PTMS	DR782728	ATCCTCGTGATGTCTGTGCC	CTGGCCCTCGTGAATTTTT	268
MHCIi	DR782864	TGCTCCTTCAGCTCCTGATT	GGTGTTGTTGGAGGTCGAGT	275
ARP2/3	bankit1234533	TGGCACAATTGACTTTCCAG	CGTCTTCACTTCGCTGTCAT	311
IgJ	GW346136	AACCTCAGACTCGTGCCATC	TGAAGGTTGTGCAGAGGTCA	318
CAL2	bankit1324554	GGAGTTCAAGGCCAAGGAG	ATGGACAAGGAGGACAGGAA	341

[a] Forward universal primer sequence: AGGTGACACTATAGAATA
[b] Reverse universal primer sequence: GTACGACTCACTATAGGGA

Table 3 Comparison of standard curve line estimators generated for each gene in the House Finch multiplexed qRT-PCR assay.

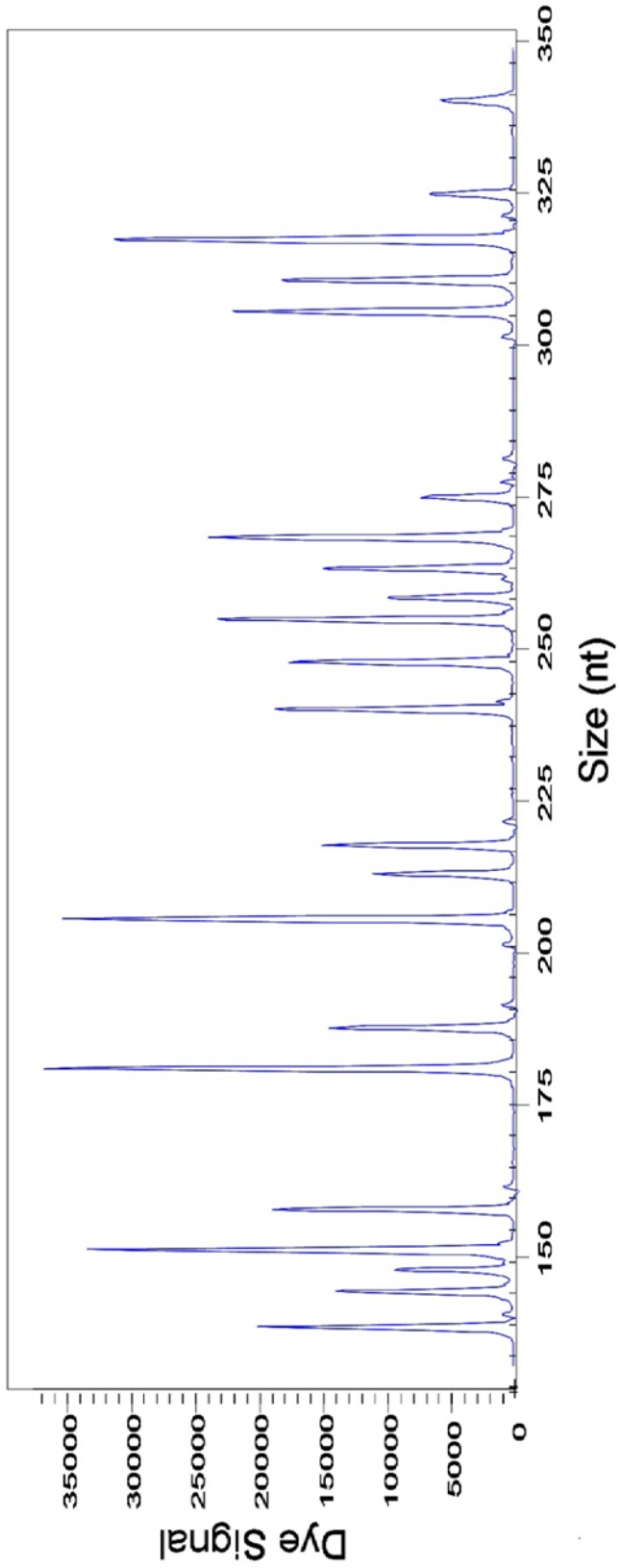
Gene	Quant Tool Line equation (r2)	Preferred Line equation according to AIC(r2)
MAP	$y = -0.076x^3 + 2.220x^2 + 1.688x$ (0.9991)	$y = (2.2 \times 10\text{-}6)x^3 - 0.001x^2 + 0.142x$ (0.9726)
TXN	$y = -1.035x^3 + 14.200x^2 - 12.939x$ (0.9969)	$y = (1.5 \times 10\text{-}6)x^3 - 0.001x^2 + 0.078x + 0.484$ (0.9627)
Ig4A	$y = -0.017x^3 + 1.910x^2 + 14.113x$ (0.9989)	$y = (4.7 \times 10\text{-}7)x^3 - 0.000x^2 + 0.064x$ (0.9665)
DSTN	$y = -0.020x^3 + 0.962x^2 + 0.527x$ (0.9988)	$y = (3.6 \times 10\text{-}6)x^3 - 0.002x^2 + 0.230x$ (0.9802)
LCP	$y = -0.023x^3 + 0.879x^2 + 2.958x$ (0.9991)	$y = (2.5 \times 10\text{-}6)x^3 - 0.001x^2 + 0.178x$ (0.9829)
PSAP	$y = -0.035x^3 + 1.457x^2 - 3.990x$ (0.9988)	$y = (3.0 \times 10\text{-}6)x^3 - 0.001x^2 + 0.2179x + 2.641$ (0.9465)
RHOA	$y = -0.141x^3 + 3.010x^2 + 3.559x$ (0.9993)	$y = (1.7 \times 10\text{-}6)x^3 - 0.001x^2 + 0.109x$ (0.9800)
SEC61	$y = -0.011x^3 + 0.596x^2 + 0.637x$ (0.9993)	$y = (4.5 \times 10\text{-}6)x^3 - 0.002x^2 + 0.283x$ (0.9848)
TCR	$y = -0.054x^3 + 1.626x^2 + 8.889x$ (0.9997)	$y = (8.0 \times 10\text{-}7)x^3 - 0.000x^2 + 0.089x$ (0.9874)
HSP90	$y = -0.051x^3 + 1.428x^2 + 4.989x$ (0.9999)	$y = (1.7 \times 10\text{-}6)x^3 - 0.001x^2 + 0.127x$ (0.9934)
ICK	$y = -0.010x^3 + 0.576x^2 + 6.146x$ (0.9998)	$y = (1.1 \times 10\text{-}6)x^3 - 0.001x^2 + 0.136x$ (0.9937)
eIF4E	$y = -0.017x^3 + 0.711x^2 + 6.277x$ (0.9998)	$y = (1.2 \times 10\text{-}6)x^3 - 0.001x^2 + 0.130x$ (0.9918)
NADH4	$y = -0.034x^3 + 1.373x^2 + 0.375x$ (0.9992)	$y = (3.2 \times 10\text{-}6)x^3 - 0.001x^2 + 0.199x$ (0.9904)
UBC7	$y = -0.117x^3 + 3.644x^2 + 6.308x$ (0.9990)	$y = (1.1 \times 10\text{-}6)x^3 - 0.001x^2 + 0.085x$ (0.9897)
NABP	$y = -0.123x^3 + 3.021x^2 - 0.491x$ (0.9982)	$y = (2.6 \times 10\text{-}6)x^3 - 0.001x^2 + 0.146x$ (0.9910)
PTMS	$y = -0.027x^3 + 1.267x^2 + 1.052x$ (0.9995)	$y = (2.9 \times 10\text{-}6)x^3 - 0.001x^2 + 0.190x$ (0.9908)
MHCIi	$y = -0.333x^3 + 5.546x^2 + 3.664x$ (0.9987)	$y = (1.3 \times 10\text{-}6)x^3 - 0.001x^2 + 0.084x$ (0.9953)
ARP2/3	$y = -0.027x^3 + 1.189x^2 + 3.326x$ (0.9999)	$y = (2.0 \times 10\text{-}6)x^3 - 0.001x^2 + 0.154x$ (0.9913)
IgJ	$y = -0.021x^3 + 1.011x^2 - 1.064x$ (0.9991)	$y = (3.9 \times 10\text{-}6)x^3 - 0.002x^2 + 0.236x + 0.806$ (0.9763)
CAL2	$y = -1.467x^3 + 15.756x^2 + 10.446x$ (0.9994)	$y = (5.7 \times 10\text{-}7)x^3 - 0.001x^2 + 0.044x$ (0.9830)

Table 4 Rank comparisons of three best-fit lines for each gene in multiplex.

Gene	Model	AICc	ΔAICc	*wi*
DSTN	x^3	102.4063	0	0.803126
	Int+x^3	105.2624	2.8561	0.19257
	Int+x^2	112.8644	10.4581	0.004304
ARP2/3	x^3	11.93277	0	0.772668
	Int+x^3	14.84648	2.91371	0.180007
	Int+x^2	17.51838	5.58561	0.047325
CAL2	x^3	59.10992	0	0.687638
	Int+x^3	60.83259	1.72267	0.290594
	Int+x^2	66.01557	6.90565	0.021768
HSP90	x^3	52.59329	0	0.681286
	Int+x^3	54.18322	1.58993	0.307667
	Int+x^2	60.837	8.24371	0.011047
ICK	x^3	58.67683	0	0.753721
	Int+x^3	61.15804	2.48121	0.217983
	Int+x^2	65.24143	6.5646	0.028296
Ig4A	x^3	61.5666	0	0.549648
	Int+x^2	62.62784	1.06124	0.323325
	Int+x^3	64.49635	2.92975	0.127027
IgJ	Int+x^3	92.93569	0	0.820066
	x^3	95.97492	3.03923	0.179427
	Int+x^2	107.715	14.77933	0.000506
LCP	x^3	94.77201	0	0.788569
	Int+x^3	97.6658	2.89379	0.18555
	Int+x^2	101.6054	6.83342	0.025881
MAP	x^3	85.31629	0	0.721226
	Int+x^3	87.73907	2.42278	0.214769
	Int+x^2	90.16029	4.844	0.064004
MHCIi	x^3	11.93855	0	0.744559
	Int+x^3	14.07816	2.13961	0.25544
	Int+x^2	38.98679	27.04824	9.96E-07
NABP	x^3	53.96058	0	0.705102
	Int+x^3	55.70417	1.74359	0.294874
	Int+x^2	74.55836	20.59778	2.37E-05
NADH4	x^3	71.42171	0	0.747743
	Int+x^3	73.59552	2.17381	0.252183
	Int+x^2	89.86057	18.43886	7.41E-05
PSAP	Int+x^3	113.0362	0	0.858479
	Int+x^2	116.6421	3.6059	0.141488
	x^3	133.3473	20.3111	3.34E-05

PTMS	x^3	67.49905	0	0.783384
	Int+x^3	70.07083	2.57178	0.216531
	Int+x^2	85.74376	18.24471	8.55E-05
RHOA	x^3	72.17634	0	0.799146
	Int+x^3	75.09607	2.91973	0.185616
	Int+x^2	80.09588	7.91954	0.015238
SEC61	x^3	110.9999	0	0.798899
	Int+x^3	113.784	2.7841	0.198579
	Int+x^2	122.5159	11.516	0.002522
TCR	x^3	53.80122	0	0.716231
	Int+x^3	56.30142	2.5002	0.205183
	Int+x^2	58.22084	4.41962	0.078586
TDX	Int+x^3	35.69285	0	0.990905
	x^3	45.16785	9.475	0.008681
	Int+x^2	51.25118	15.55833	0.000415
eIF4E	x^3	64.44427	0	0.766043
	Int+x^3	67.34391	2.89964	0.179723
	Int+x^2	69.74014	5.29587	0.054234
UBC7	x^3	30.98168	0	0.765229
	Int+x^3	33.35268	2.371	0.233849
	Int+x^2	44.42313	13.44145	0.000923

Fig. 1 Electropherogram of fluorescence-labeled RT-PCR products synthesized with primers described in Table 1.



IV. RELATIVE PLUMAGE COLOR PREDICTS GENE EXPRESSION IN THE TRACHEA OF HOUSE FINCHES INFECTED WITH *MYCOPLASMA GALLISEPTICUM*

Abstract

Sexually dimorphic ornamentation is generally thought to arise as the product of male-male competition over mates or female mating preferences. While it is now accepted that female choice can drive the evolution of such ornamental traits, what, if any, benefits females gain from such choice is often unclear. In instances where tangible benefits can not be identified, trait association with indirect benefits, like a male's genetic or physical quality, are usually invoked to explain female preference for ornamentation. However, the mechanisms underlying many hypothesized genetic or physical quality associations with ornamentation remain largely unexplored. Here I attempt to elucidate molecular associations between feather color and response to disease at the level of gene expression in the trachea of the house finch (*Carpodacus mexicanus*), a North American songbird with sexually selected ornamental plumage coloration, experimentally infected with the bacterium *Mycoplasma gallisepticum* (MG). I examined birds originating from two distinct populations, Alabama and Arizona, having a 12-yr history of exposure to the disease and no history of exposure to the disease, respectively. To better understand the mechanisms through which ornamental male plumage color relates to response to infection, I compared trachea gene expression of redder and yellower finches infected with MG. Using a candidate gene approach, I found that Prosaposin (PSAP) was significantly up-regulated in the trachea of yellower (less ornamented) house finches infected with MG when compared to redder (more ornamented) birds infected with MG. Contrary to predictions, however, I did not

find a relationship between the expression of PSAP and severity of infection. It is, therefore, still unclear what if any functional role PSAP might play in response to MG infection.

Introduction

A widespread observation in animals is that the degree of elaboration of ornamental traits is positively associated with various aspects of performance. For instance, carotenoid-based plumage coloration in birds predicts timing of molt (Hill and Montgomerie 1994), rate of feather growth (Hill and Montgomerie), nest provisioning (Hill 1991, Germain 2010), and problem solving (Matoes 2011). In recent years carotenoid ornamentation has also been shown to relate to various aspects of immune performance, including measures of immunocompetence and resistance to disease (Mougeot 2008, Dawson and Bortolotti 2006, van Oort and Dawson 2005). While a few studies have found associations between ornamentation and recovery from (Lindström & Lundström 2000, Hill & Farmer 2005) and resistance to (Roulin et al. 2005) pathogens, the mechanisms that underlie these associations between performance and ornamentation remain largely unresolved (Hill 2011).

One way to identify candidate molecules functionally relating immune response and ornamentation is to measure and compare gene expression in highly and poorly ornamented diseased individuals. Genes that are differentially expressed in individuals undergoing an identical immune challenge that differ with respect to ornamentation may be directly or indirectly related to ornament expression. For example, if a gene is up-regulated in response to infection with a pathogen in birds with redder, higher quality, plumage color, this could be because that gene is functionally involved in both pathogen resistance and metabolic carotenoid conversion or deposition into feathers (a direct relationship). Alternatively, this association

could simply be a product of the condition of the bird. In this case plumage color correlates with immune response because, even on a controlled diet, redder individuals are generally healthier and in better condition than are yellower individuals and, thus, one could reasonably expect them to respond to pathogens differently (an indirect relationship). While patterns of gene expression are phenotypic traits, identification of expression differences in candidate genes can both provide information regarding relationships between the selected genes in response to infection, as well as providing evidence leading to the investigation of the potential genetic differences underlying gene expression.

Here I studied patterns of gene expression of male house finches (*Carpodacus mexicanus*) that I experimentally infected with the bacterial pathogen *Mycoplasma gallisepticum* (hereafter referred to as MG). House Finches are sexually dichromatic such that females are drab brown and gray while males have bright carotenoid-based coloration (Inouye et al. 2001) on their heads, breasts, and rumps. This carotenoid-based coloration tends to be reddish in most males, but it varies from pale yellow to bright scarlet red. Previous lab and field experiments have demonstrated female preference for the reddest male house finches (Hill 1990, 1991, 1994), and redder males tend to provide more food to their incubating mates and to young in the nest (Hill 1991, 2002). House finch plumage coloration is affected by access to carotenoid pigments (Hill 1992, 1993), general nutrition (Hill 2000), and parasite load (Brawner et al. 2000, Hill et al. 2004) during molt. Importantly, house finches ingest carotenoid pigments in a precursor form that they must then metabolically convert into derivative forms prior to feather deposition (Hill 2002). Although the molecular mechanisms underlying carotenoid conversion in birds are not yet clear, it is likely that the process of ornament production is influenced not only by diet, but also by genetics and physiological state, including general stress and health (Hill 2011).

MG is a respiratory pathogen in house finches and typically causes conjunctivitis symptoms (Ley et al. 1996). In the mid-1990s, an epizootic of MG seriously impacted populations of house finches in the eastern half of the United States, resulting in the death of tens of millions of house finches (Nolan et al. 1998, Hochachka & Dhondt 2000, Dhondt et al. 2005). Previously known only as a poultry infection, MG spread through populations of house finches across the eastern US (Nolan et al. 1998, Hochachka & Dhondt 2000, Dhondt et al. 2005).

Following the initial outbreak of MG in house finches in Alabama, population-wide male plumage color showed a significant shift towards the redder plumage color that is preferred by females in mates (Hill 1990, 1991, 1994, 2002), suggesting a selective event for more highly ornamented males. Subsequent controlled experimental studies have further found that infected male finches grow significantly yellower feathers than males not infected with MG (Brawner et al. 2000, Hill et al. 2004) and that redder males recover from MG infection faster than do yellower males (Hill & Farmer 2005). These studies suggest that feather color indicates a male's infection status during molt as well as his ability to fight MG infection. In a previous microarray study (Chapter 2), I identified gene expression patterns in the spleens of redder and yellower birds infected with MG when the birds originated from western US populations previously unexposed to the bacterial pathogen (Arizona). The gene expression patterns detected for redder Arizona birds were consistent with increased resistance, while patterns detected for yellower Arizona birds were consistent with an increased susceptibility to MG (Chapter 2), suggesting the existence of within-population variation in resistance to a novel bacterial pathogen that is associated with plumage color.

Here I further investigate gene expression differences in another immune tissue, the trachea, of redder and yellower finches in response to MG infection. My previous studies addressed

response by infected finches in the spleen, which is a part of the lymphatic immune system and a site in general for both humoral and cell-mediated immune responses. The trachea, on the other hand, is a primary site of MG localization across its host species, is part of the mucosal immune system (Kothlow and Kaspers 2009) and the tracheas of chickens infected with MG have been shown to respond to MG infection with extensive lymphocyte infiltrations and lymphoproliferation (Gaunson et al. 2006).

Finches in the current study originated both from eastern U.S. populations (Alabama) that have been exposed to MG since 1995 (Nolan et al. 1998), and from western U.S. populations (Arizona) that had never been exposed to MG at the time of the experiment (Toomey et al. 2010). I measured individual expression levels of 10 candidate genes using multiplexed quantitative amplifications of trachea tissues. To identify genes whose expression in the trachea is primarily associated with plumage color rather than population history with MG, I first test whether there are transcriptional differences in infected birds between populations. When population of origin does not significantly affect gene expression, I ask, regardless of population history with the pathogen, whether red and yellow birds infected with MG differ in their response at the level of gene expression. I then test the functional significance of any gene expression differences by quantifying MG load in the conjunctivae of infected finches; when differences in gene expression are reflective of a bird's ability to fight MG, I predict that expression levels will be related to MG load.

Materials and Methods

Capture, housing and experimental infection

Birds were captured using mist nets or wire mesh cages placed around feeders. Following capture, birds were immediately transported by plane from Arizona and by car within Alabama, and established in aviaries at Auburn University, Alabama. Birds were housed in cages as pairs for the duration of their period in captivity. Housing and care of birds are described in detail in Chapters 1 & 2. Birds from Alabama and Arizona were kept in separate rooms for the first month to check that they were MG-free. Following quarantine, birds were measured and blood sampled using brachial venipuncture (~60 µl of whole blood) and examined for exposure to MG using serum plate agglutination assay (SPA) (Luttrell et al. 1996) and amplification of MG DNA from choanal and conjunctival swabs (Roberts et al. 2001). No birds used in any part of the experiment were found to have been previously infected with MG.

Birds were infected via ocular inoculation with a total of 20 µl of culture containing $1x10^4$ to $1x10^6$ color changing units/ml of an early 2007 Auburn MG isolate (BUA #243). After exposure, birds were monitored daily for disease onset and progression of symptoms. All experimentally infected birds tested positive for MG DNA in their choanal cleft 3 and 14 days post-inoculation and all were seropositive after two weeks. Fourteen days post treatment, birds were euthanized under license. The spleen, tracheas and the eyes/conjunctivae from all euthanized birds were immediately removed, stored in RNAlater (Ambion) and placed at -80°C.

RNA extractions

I extracted total RNA from approximately 10 mg of each individual's homogenized trachea tissue using Qiagen RNeasy fibrous tissue miniprep spin columns, including on-column digestion of genomic DNA according to the manufacturers' protocols (Qiagen, Valencia, CA). I

determined the quantity of purified total RNA using a Nanodrop spectrophotometer. All RNA extracts were stored at -80ºC until further processing.

Color Analysis

I measured reflectance spectra of collected feathers across the avian visual range (300-700 nm) following standard procedures (Siefferman & Hill 2003, Perrier et al. 2002, Quesada & Senar 2006) to collect raw spectral data using an Ocean Optics S2000 spectrometer (Dunedin, FL). Following (Hill 2002, Hill & Farmer 2005), I used hue as the most relevant descriptor of male House Finch color. I quantified hue as the wavelength of 50% reflectance (λR50) located at the midpoint between the maximum and minimum reflectance contained within the visible spectrum (Pryke et al. 2001).

Multiplexed quantitative RT-PCR analysis of trachea

I measured transcriptional changes in the trachea of each infected bird at 20 genes using multiplexed quantitative real-time amplifications (details of assay are provided in detail in Chapter 3). Of the 20 genes amplified in the multiplex, I *a priori* selected a subset of 10 genes for analysis because they could be assigned to at least one of the following three categories: 1) they were found to be statistically significantly differentially expressed between infected yellow birds and controls from AZ, 2) they were qualitatively different between infected yellow and infected red birds from AZ, and/or 3) they are predicted to be involved in immune response (Table 1).

Quantification of MG in eye conjunctivae

As detailed in Chapter 1, I randomly selected one eye along with its conjunctivae from each bird. I isolated total genomic DNA from both MG and house finches using the Qiagen AllPrep DNA/RNA Mini Kit. I then quantified MG by running RT-qPCR assays on the *mgc2* gene (Grodio et al. 2008). I controlled for variation in amount of starting material by amplifying the house finch *rag1* gene. I generated a standard curve for both genes using 100, 50, 25, 10, 1 and 0.1 g of genomic DNA which allowed for an estimation of the relative amount of MG between individuals. Reactions were run on an ABI Prism 7500 (Applied Biosystems). Details of reactions and cycling parameters are given in Chapter 1. Automatic threshold settings were used for sample analysis.

Statistical Analyses

I used Spearman's rank correlations to explore relationships between gene expression in the trachea of the 10 House Finch candidate genes and to determine the potential for correlated responses. Gene expression data was then transformed to a normal distribution when possible (6 out of 10 genes; see Table 1)**.** Normality was evaluated using the Kolmogorov-Smirnov Goodness-of-Fit test**.** However, due to small sample size and the inherently non-normally distributed nature of the expression of some genes, in some cases I could not transform expression data to a normal distribution (n = 4). In such cases, I converted the data to a binomial distribution, where 0 = not detectably expressed and 1 = expressed.

Due to small overall sample sizes I would have had very low power to identify relationships between color and gene expression if population were to be included in the statistical models. To determine whether it was appropriate for gene expression data to be pooled across populations,

therefore, I first used generalized linear models for normalized variables or Fisher's exact tests for binomial variables to identify whether gene expression was significantly related to population of origin. When population was a significant predictor of gene expression, I excluded these genes from all further analyses. However, when population was not a significant predictor of gene expression, I pooled data across populations within color groups. Birds were assigned to plumage color groups within the context of their population of origin and then groups having relatively longer (i.e., redder) and shorter (i.e., yellower) wavelength hues given their population of origin were pooled.

For the remaining genes whose expression could be transformed to a normal distribution, I used generalized linear models to test whether 1) gene expression was related to plumage color, and 2) the quantity of MG in the eye conjunctiva was related to expression of genes whose expression in the trachea was associated with plumage color. Models were evaluated based on Wald χ^2 values. For the remaining genes whose expression was classified as binomial, I tested 1) and 2) using Fisher's exact tests because in each case the frequency of one category was 5 or less. All statistical analyses were performed using SAS v. 9.2 (SAS Institute, Cary, NC).

Results

Plumage color

The hue of each bird examined with respect to candidate gene expression in the trachea was ranked within the context of its population of origin when assigning plumage color groups and then individuals were pooled from each population, such that redder birds had relatively long wavelength hues for their population of origin (i.e., redder: AZ birds: n = 6, $\overline{x}$ = 613 nm, range =

610-616 nm; AL birds: n = 5, $\overline{x}$ = 607 nm, range = 604-610 nm) and yellower birds had relatively short wavelength hues for their population of origin (i.e., yellower: AZ birds: n = 5, $\overline{x}$ = 582 nm, range = 567-599 nm; AL birds: n = 7, $\overline{x}$ = 591 nm, range = 584-598). Mean hue between the subset of birds used for this experiment was similar (596 nm for the Alabama population and 597 nm for the Arizona population), but the hue of Arizona birds ranged across 49 nm in contrast to the hue of Alabama birds which only ranged across 26 nm (Fig. 1). I compared gene expression in the trachea of 11 infected birds with redder hues and 12 infected birds with yellower hues.

Expression of candidate genes in relation to population of origin and relative plumage color

Spearman rank correlations were performed on each of the 10 house finch genes chosen as candidates for this study (Table 2). I found highly significant relationships between the expression of several of the genes, particularly those chosen for inclusion based on their predicted function in immune response (i.e., MHCIi, Tcrb, IgJ, and Ig4A).

Population of origin significantly predicted expression of Hsp90 (Table 3), while the nine other genes examined were not significantly affected by whether birds were from Arizona or Alabama. Hsp90 was therefore excluded from plumage color analyses.

The remaining nine candidate genes were analyzed in relation to plumage color (summarized in Table 3). Gene expression was related to plumage color, regardless of population of origin, in one of the nine genes examined: prosaposin (PSAP).

Candidate gene expression and MG quantity

The quantity of MG measured in the eye conjunctiva of house finches was not significantly associated with amount of prosaposin expressed in the trachea ($\chi^2 = 0.45$, $p = 0.50$; Fig. 2).

Discussion

In Chapter 1 I showed that house finch populations with differing histories of exposure to MG have different responses to infection at the level of gene expression in the spleen. In Chapter 2, I examined male house finches originating exclusively from the Arizona population with no prior history of exposure to MG. In that study, I found differences in gene expression of redder and yellower males infected with MG when each was directly compared to controls. Specifically, 18 genes were identified as differentially expressed between infected yellow birds and controls while only one gene was identified as differentially expressed between infected red birds and controls. Results from that study suggested that redder Arizona birds exhibited a more adaptive response than did yellower Arizona birds, supporting the hypothesis that female house finches prefer redder males because these males are signaling their ability to respond to infectious pathogens such as MG. Here I measure expression of candidate genes in the trachea, a tissue associated with mucosal immunity. This tissue is particularly important in MG infections because MG localizes to the mucosal lining of the respiratory tract and is found in the lining of the trachea of infected birds (Kothlow & Kaspers 2009). Examining gene expression between the Alabama and Arizona populations, I found that, of the 10 candidates chosen for investigation, nine genes were not significantly different between the populations. That all 10 genes previously found to be significantly differentially expressed with respect to population of origin in the spleens of these birds were not also differentially expressed in the trachea suggests either that history of exposure to this disease did not have a major effect on response in the trachea, or that

genes important for immunity in the spleen may not be important in the trachea. However, assuming that these genes do bear some sort of functional implications in the trachea, among the nine that were not different between populations, one gene, prosaposin, was found to be significantly differentially expressed between redder and yellower birds when samples from the two populations were combined.

Prosaposin, or PSAP, is a ubiquitously expressed precursor glycoprotein which is post-translationally cleaved into Saposins A, B, C, and D by partial proteolysis (Kishimoto et al. 1992, Azuma et al. 1998). Because cleavage is post-translational I was unable to determine which, if any, of the saposins are being preferentially up-regulated or if PSAP itself were the final product in the house finch trachea. In general, the saposins are involved in the degradation of glycosphingolipids into sphingosine and fatty acids within lysosomes, but PSAP itself also exists as a mature secretory protein and a membrane protein (Kishimoto et al 1992, Sun et al 1994).

In a study of gene expression in lab strains of mice, PSAP has been identified as being generally highly expressed in secretory epithelial cells of certain tissues, including the trachea (Sun et al 1994). In addition, several immune cell types and tissues, including the Harderian glands, macrophages in the lymph nodes, the spleen and the thymus, also showed relatively high levels of expression (Sun et al 1994). In a recent study examining transcriptional responses by macrophage cell lines to infection with *Brucella spp.*, Covert et al. (2009) identified PSAP transcripts as down-regulated in infected cells when compared to uninfected cells. *Brucella spp.* are zoonotic bacterial pathogens that localize intracellularly via macrophage phagocytosis and then are capable of subverting host cell antimicrobial defense mechanisms and thus avoid the host humoral immune response altogether (Baldwin et al. 2006, Covert et al. 2009).

In the last decade, several studies have examined SNPs, or Single Nucleotide Polymorphisms, located within the gene for PSAP in domestic poultry as possible QTLs (Quantitative Trait Loci) for predicting an individual's response to vaccination and bacterial challenge (Liu and Lamont 2003, Kramer et al. 2003, Ahmed 2010). Liu and Lamont (2003) identified a silent mutation in the PSAP gene that was significantly associated with lower bacterial load in the spleen of 6 day old chickens that had been challenged with the bacteria when 1 day old, although this allelic variant was not significantly related to bacteria in the caecum (the primary site of *S. enteriditis* horizontal transmission as it is primarily passed through ingested faeces) or to *S. enteriditis* antibody titers. The authors propose that, because Salmonella can survive in host cells by modifying phagosomes (which employ lysosomes in the destruction of *Salmonella*), PSAP may play a direct role in *Salmonella* infection (Liu and Lamont 2003). In a follow-up study, Kramer et al. (2003) challenged 3 week old chickens for 1 week with *S. enteriditis* and found that the bacterial load in the caecum, but not the spleen or liver, was reduced in animals with this PSAP allelic variant. This variant, however, only explained between 5-8% (depending on the breed of chicken) of the phenotypic variation in the number of bacteria in the caecum (Kramer et al. 2003). The authors suggest that, because it is a silent mutation, the effect of this SNP in the PSAP gene might be due to linkage to functional polymorphisms in nearby genes, although the actual mechanism is still unknown (Kramer et al. 2003).

Given the significant difference in expression of PSAP in the trachea, such that yellower birds express more PSAP in response to infection than do redder birds, I predicted that expression of this gene should have a direct relationship with severity of illness in response to MG infection. I measured illness severity by counting the number of MG cells in the eye and

conjunctiva of each bird using qPCR and compared a bird's total count of MG to its expression of PSAP in the trachea. Somewhat surprisingly I did not find a relationship between the production of PSAP transcripts and the amount of MG in a bird's eye. It is possible that if this gene actually is important in house finch response to MG infection, then it does not act at the level of quantity of infection. One possibility is that the effects of PSAP are not evidenced by the amount of MG but rather by the virulence of MG. This is especially relevant for expression in the trachea, a major site of MG localization, where finch epithelial cells and the MG pathogen directly interact. At this point, however, it is unclear what, if any, significance there is in the difference in house finch expression of PSAP 14 days after exposure to MG. One other real possibility is that, as was found in the QTL studies of chicken response to *Salmonella*, PSAP contributes only a small proportion to the overall response by house finches to MG and that larger sample sizes than those presented here will be needed to detect the relationship between PSAP expression and quantity of MG.

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Table 1 List of genes included in House Finch gene expression analyses and brief justification. Primer sequences are given in Table 1 of Chapter 3.

Gene Name (Abbreviation)	Accession No.	Reason for Inclusion[a]	Transformation	Distribution
Destrin (Dstn)	NM_205528.1	1, 2	ln	Normal
Heat shock protein 90(Hsp90)	XM_002200572.1	1, 2	ln	Normal
Immunoglobulin 4A (Ig4A)	XM_002194781.1	3	ln	Normal
Immunoglobulin J (IgJ)	DQ213324.1	3	N/A	Binomial
MHC class II-associated invariant chain Ii (MhcIi)	DQ215319.1	1, 2, 3	N/A	Binomial
Prosaposin (Psap)	DQ214627.1	1, 2	ln	Normal
SEC61γ (Sec61)	XM_002198448.1	1, 2	ln	Normal
T cell receptor β (Tcrb)	AF068228.1	1, 2, 3	N/A	Binomial
Translation Initiation Factor 4E (eIF4E)	DQ213184.1	1, 2	inverse ln	Normal
Ubiquitin C (Ubc)	DQ216247.1	1, 2	N/A	Binomial

[a] House Finch (HOFI) genes were selected for investigation from the plex described in Chapter 3 for at least two of three reasons:

1) they were found to be statistically significantly differentially expressed between infected yellow birds and controls from AZ,
2) they were qualitatively different between infected yellow and infected red birds from AZ, and/or
3) they are predicted to be involved in immune response.

Table 2 Spearman rank correlations of expression values for 10 candidate genes measured in the trachea of house finches infected with *Mycoplasma gallisepticum* for 14 days (n = 23). All $p < 0.05$ are bolded.

	DSTN	HSP90	Ig4A	IgJ	MHCIi	PSAP	SEC61	TCRB	eIF4E
HSP90	0.202 *0.356*	1.000							
Ig4A	-0.073 *0.740*	0.424 ***0.044***	1.000						
IgJ	0.012 *0.957*	-0.541 ***0.008***	-0.384 *0.070*	1.000					
MHCIi	0.224 *0.304*	-0.194 *0.376*	-0.238 *0.274*	0.734 ***0.001***	1.000				
PSAP	0.130 *0.553*	0.117 *0.596*	0.534 ***0.009***	-0.196 *0.371*	-0.134 *0.541*	1.000			
SEC61	0.545 ***0.007***	-0.087 *0.693*	-0.210 *0.335*	0.446 ***0.033***	0.604 ***0.002***	0.093 *0.673*	1.000		
TCRB	0.196 *0.371*	0.035 *0.876*	-0.163 *0.457*	0.542 ***0.008***	0.707 ***0.001***	-0.418 ***0.047***	0.274 *0.206*	1.000	
eIF4E	0.462 ***0.026***	-0.054 *0.806*	-0.127 *0.562*	0.468 ***0.024***	0.733 ***0.001***	0.122 *0.581*	0.580 ***0.004***	0.485 ***0.019***	1.000
UBC	0.299 *0.165*	0.341 *0.111*	0.508 ***0.013***	-0.226 *0.299*	0.125 *0.571*	0.322 *0.134*	0.352 *0.100*	-0.097 *0.660*	0.123 *0.578*

Table 3 Results of generalized linear models and Fishers exact tests examining whether population of origin (n = 12 exposed to *Mycoplasma gallisepticum*; n = 11 unexposed to *Mycoplasma gallisepticum*) or plumage color (n = 11 red; n = 12 yellow) predicts expression of candidate genes by birds experimentally infected with *Mycoplasma gallisepticum* for 14 days.

	Population of Origin		Plumage Color[a]	
Gene	χ^2	*p*	χ^2	*p*
DSTN[c]	1.02	*0.31*	1.36	*0.24*
HSP90[c]	5.04	***0.03****	N/A	N/A
Ig4A[c]	1.01	*0.31*	2.59	*0.11*
IgJ[b]		*0.22*		*1.00*
MHCIi[b]		*1.00*		*0.67*
PSAP[c]	2.69	*0.10*	5.50	***0.02****
SEC61[c]	0.01	*0.92*	0.02	*0.90*
TCRB[b]		*0.40*		*1.00*
eIF4E[c]	2.36	*0.13*	0.02	*0.89*
UBC[b]		*1.00*		*0.68*

[a] Where test statistics are given as N/A, this gene was significantly different based on population of origin and, therefore, samples could not be pooled across color groups. When this occurred I did not attempt to predict gene expression of the gene using plumage color.
[b]Fisher's exact test used for binomially distributed response variables.
[c]Generalized linear models used for normally distributed response variables.

Fig. 1 Comparison of rump hues of male house finches originating from eastern U.S. populations (Alabama) that have been exposed to MG since 1995, and from western U.S. populations (Arizona) that had never been exposed to MG at the time this study was conducted. Boxplots show means (plus signs), medians (horizontal lines within boxes), 25th and 75th percentiles (lower and upper bounds of boxes, respectively), and minimum and maximum values (extreme lower and upper bars, respectively).

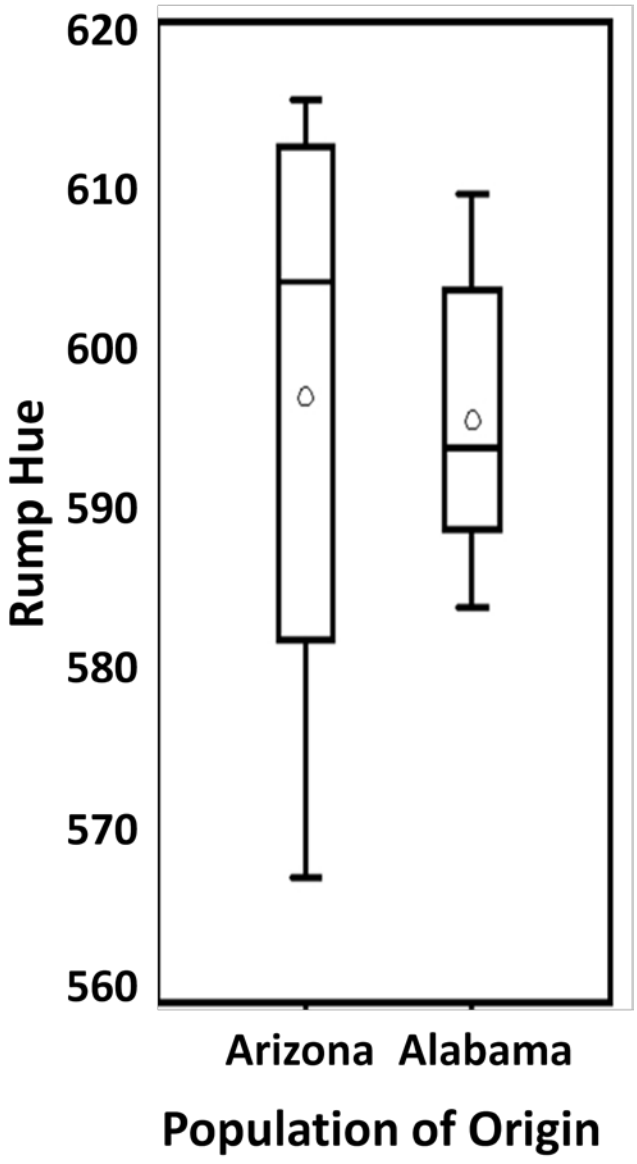
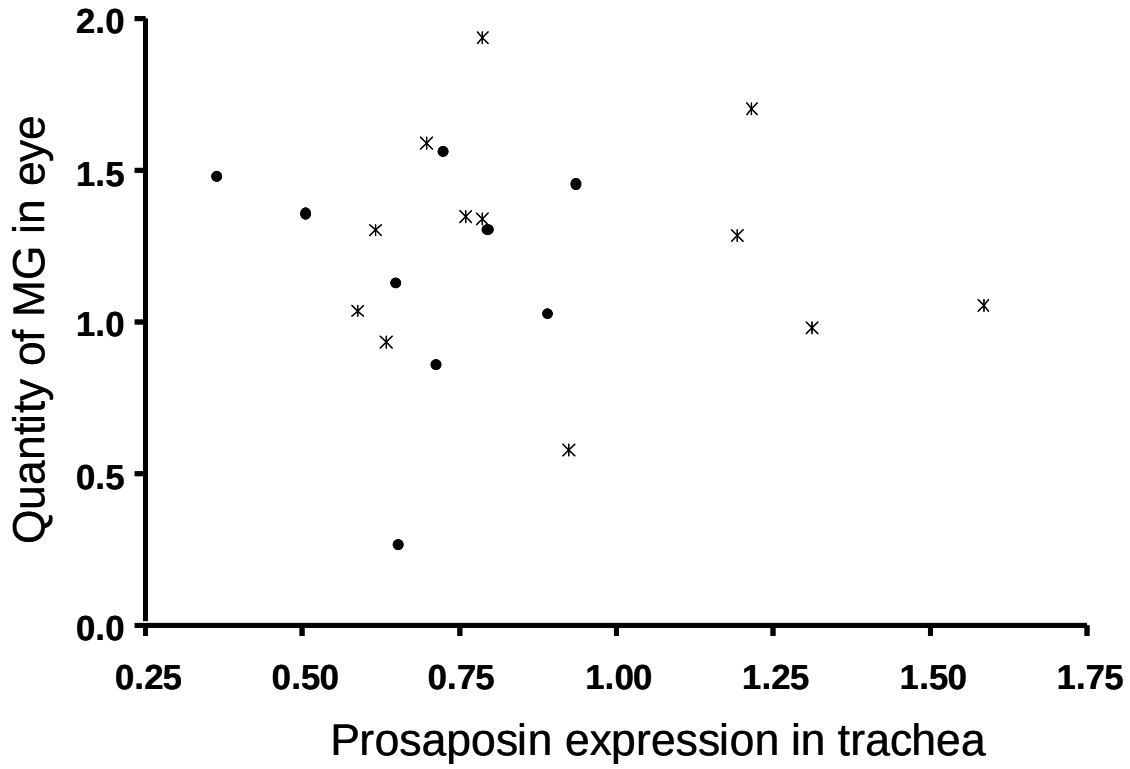


Fig. 2 Relationship between the amount of *Mycoplasma gallisepticum* (MG) present in the eye of experimentally infected House Finches and expression of prosaposin in the trachea of those individuals 14 days after infection. Solid circles represent data for red birds; asterisks represent data for yellow birds.



Appendix 1

Online Gene Functions

Among the known genes that were significantly differentially expressed (Table 1), I detected 10 that have been identified with primary immune function and hence could have been directly involved in the immune response to MG. For example, of the 6 genes differentially expressed in comparisons 1 and 2 (Fig. 2a): **MHC class II-associated invariant chain Ii** plays a role in the assembly of MHC class II molecules, which serve to recognize foreign peptides originating from the degradation of extra-cellular parasites (Bertolino & Rabourdin-Combe 1996); **lectin galactoside-binding soluble 2 protein** (galectin 2) belongs to a family of proteins differentially expressed in various immune cells and up-regulated during infections (Rubinstein et al. 2004); **galectins** are involved in the regulation of cellular immune responses and immune cell homeostasis (Liu 2005), and galectin 2 is thought to control inflammation and regulate activated CD8+ T cells (Loser et al. 2007); **programmed death ligand 1** plays a key role in regulating T cell activation and tolerance (Brown et al. 2003, Greenwald et al. 2005); **neutrophil cytosolic factor 4**, 40kD encodes for a subunit of the superoxide-producing phagocyte NADPH-oxidase and plays an important role in phagocytosis-induced superoxide production, an essential mechanism in host innate immune defence (Matute et al. 2009); **T-cell immunoglobulin and mucin domain containing 4** is primarily expressed on antigen-presenting cells and can play a role in T-cell activation and help sustain an ongoing immune response (Rodriguez-Manzanet et al. 2009), as well as serve to mediate the engulfment of apoptotic cells (Kobayashi et al. 2007), thereby maintaining tolerance and preventing inflammation and autoimmunity against intracellular antigens released from the dying cells (Savill & Fadok 2000). By contrast, the one

immune gene up-regulated in comparison 4, **hCG40889** (complement factor H) is secreted in the plasma protein to regulate complement-mediated immunity, which plays a key role in microbial killing. By preventing excessive activation of the complement cascade, it participates in protecting host cells and tissues (de Cordoba & de Jorge 2008, Boon et al. 2009).

I also identified 6 genes with auxiliary immune function, all of which were down-regulated in the infected unexposed versus exposed populations. It is known that MG is exposed to host reactive oxygen species (ROS) (Jenkins et al. 2008), and that the generation of ROS by phagocytic cells during oxidative bursts is an important antibacterial mechanism (Fang 2004). ROS are free radicals (e.g., superoxide O_2^-, hydroxyl radicals OH, hydrogen peroxide H_2O_2) that are produced at high levels to kill internalized pathogens (Swindle & Metcalfe 2007). **Squalene epoxidase** catalyses the first oxygenation reaction of cholesterol biosynthesis (Abe et al. 2000), and has been suggested to play a role in the production of ROS, possibly via effects of sterols on the localization of NADPH oxidase (Pose et al. 2009).

The action of ROS is non-specific and so as they accumulate, they damage host tissues and pathogens indiscriminately, e.g. by inducing DNA or cell damage through lipid peroxidation (Droge 2002, Valko et al. 2007). To minimize such costs, hosts have developed ROS scavenging mechanisms, such as the enzyme superoxide dismutase, which catalyzes the dismutation of superoxide (O_2^-) into oxygen (O_2) and hydrogen peroxide (H_2O_2). **Thioredoxin**, an oxidoreductase system induced by oxidative stress, has important antioxidant activities (Nordberg & Arner 2001), and both ROS and thioredoxin can also influence downstream immune functions through the regulation of transcription factors and cytokines (Sen & Packer 1996, Bubici et al. 2006). **Spermidine/spermine N1-acetyltransferase** is one of the main enzymes responsible for the regulation of intracellular polyamine (Casero & Pegg 1993). This

protein can be induced by the pro-inflammatory cytokine: tumor necrosis factor-alpha (TNFα), to help maintain normal cellular physiology under inflammatory stress (Babbar et al. 2007). Both spermine and spermidine exert protective functions of the cell under oxidative stress (Rider et al. 2007).

Rho GTPase is involved in several signal transduction pathways and plays an important role in the regulation and coordination of the innate immune response (reviewed in Bokoch & Knaus 2003, Scheele et al. 2007). Rho GTPase proteins are involved in Toll-like receptor signalling, a key line of defense against microbial pathogens (Aderem & Ulevitch 2000). They also form a subunit of the NADPH oxidase complex where they regulate the formation of ROS during oxidative bursts (Kao et al. 2008). Another important role of Rho GTPase proteins is their implication in actin and microtubules regulation and cytoskeletal rearrangements mediating leukocytes chemotaxis and motility, phagocytosis as well as lymphocyte cytotoxicity (Cicchetti et al. 2002, Khurana & Leibson 2003, Scott et al. 2005). The higher expression of cytoskeletal elements in infected birds from the exposed-population compared to infected birds from the unexposed-population is consistent with the up-regulation of Rho GTPase in those former individuals. Indeed, beta-actin is one of the two isoforms present in the cytoplasm and actin polymerization and depolymerization may be driven by actin related protein and destrin (Yeoh et al. 2002).

Lymphocyte cytosolic protein (L-Plastin) has been shown to stabilize actin filaments during T lymphocyte migration (Samstag et al. 2003), while the interaction between actin filaments and myosin, and the phosphorylation of myosin regulatory light chain, generate the contractile force necessary for cell migration (Mizutani et al. 2006).

Ubiquitin C targets cellular proteins for degradation in the proteasome (Varshavsky 2003). Ubiquitination can however also be reversible and play a role in the activity and localization of proteins, in signalling pathways, as well as in the initiation of an immune response (for a review see Boyer & Lemichez 2004). The involvement of ubiquitin in immunity may be mediated through the specific degradation of inflammatory inhibitors (Silverman & Maniatis 2001), through the regulation of the tumor-necrosis factor receptor-associated factor 6 important in Toll-like receptor signalling (Deng et al. 2000), through the endocytosis of antibody-antigen complexes by Fc-receptors (Booth et al. 2002, Yamakami et al. 2003), or even through the generation of antigenic peptides important in MHC signalling (Goldberg et al. 2002).