Behavioral Ecology Advance Access published May 7, 2015

Behavioral Ecology

The official journal of the ISBE International Society for Behavioral Ecology

Behavioral Ecology (2015), 00(00), 1–11. doi:10.1093/beheco/arv055

Original Article

Plumage color and pathogen-induced gene expression in a wild bird

Susan L. Balenger,[a] Camille Bonneaud,[b] Stephen A. Sefick,[a] Scott V. Edwards,[c] and Geoffrey E. Hill[a]

[a]Department of Biological Sciences, Auburn University, AL 36849, USA, [b]Centre for Ecology and Conservation, University of Exeter, Cornwall TR10 9FE, UK, and [c]Department of Organismic Biology, Harvard University, MA 02138, USA

Received 22 August 2014; revised 25 March 2015; accepted 29 March 2015.

Ornamentation is hypothesized to signal the capacity of an individual to cope with environmental challenges. At the molecular level, organisms respond to their environments largely by altering gene transcription, but the transcriptional responses linking ornamentation and disease resistance are virtually unstudied. In the house finch (*Haemorhous mexicanus*), carotenoid coloration displayed by males is important in female choice, and plumage redness predicts a male's ability to recover from bacterial infection with *Mycoplasma gallisepticum*. To investigate the molecular mechanisms underlying links between ornamentation and disease response, we experimentally infected wild finches and used microarrays to identify genes that were differentially expressed in males with redder and yellower plumage coloration. Sixteen candidate genes were then investigated across individuals. Principal components analysis revealed that infection induced a marginally significant decrease in 11 genes, several with primary immune functions. The treatment by plumage color interaction effect was largely driven by greater disease-induced changes in expression of *heat shock protein 90* and *ubiquitin c* (the second principal component) by red birds than by yellow birds. In a direct comparison of disease severity between infected birds, plumage color on its own did not significantly predict disease symptoms. However, in multivariate models, expression of genes loading on the second principal component, plumage color, and their interaction were significant predictors of the severity of disease symptoms. Our observations demonstrate links between ornamentation and expression of genes related to disease resistance; we suggest that male plumage color may reflect functionally associated differences in inducibility or plasticity of gene expression in this species.

Key words: **carotenoid coloration, *Haemorhous mexicanus*, Hamilton–Zuk hypothesis, house finch, *Mycoplasma gallisepticum*.**

INTRODUCTION

The indicator model of sexual selection proposes that ornamental traits display reliable information about individual condition (Andersson 1994), and the theory of parasite-mediated sexual selection posits specifically that ornament quality signals disease resistance (Hamilton and Zuk 1982; Balenger and Zuk 2014). Feather coloration is frequently studied in relation to parasites, and many empirical studies have found associations between degree of parasitism and brightness of feathers (reviewed in Hill 2006). Carotenoid pigmentation, which creates most red and yellow coloration in birds (McGraw 2006), is particularly intriguing with regard to signaling parasite resistance because carotenoids are implicated in mediating oxidative stress (Alonso-Alvarez et al. 2008) and immunocompetence (Blount et al. 2003) and have been proposed to be responsive to the vitamin A and redox state of an organism (Hill and Johnson 2012; Johnson and Hill 2013) and associated with the efficiency of cellular respiration (Hill 2014).

Male house finches (*Haemorhous mexicanus*) have variable red-to-yellow carotenoid pigmentation on their head, breast, and rump feathers. Female house finches prefer redder males as mates (Hill 1990, 1991), and male feather coloration is condition dependent, shaped by carotenoid access (Hill 1992; Hill et al. 2002), general nutrition (Hill 2000), and parasite exposure during feather growth (Thompson et al. 1997; Brawner et al. 2000; Hill et al. 2004). 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). In a survey of morphological characteristics of house finches in an eastern population before and after the initial MG epidemic,

Address correspondence to S. L. Balenger, who is now at 1987 Upper Buford Circle, Department of Ecology, Evolution, and Behavior, Saint Paul, MN 55108, USA. E-mail: sbalenge@umn.edu.

© The Author 2015. Published by Oxford University Press on behalf of the International Society for Behavioral Ecology. All rights reserved. For permissions, please e-mail: journals.permissions@oup.com

Downloaded from http://beheco.oxfordjournals.org/ by guest on May 8, 2015

Nolan et al. (1998) found that red males survived the epidemic better than yellow males, resulting in directional selection on plumage color in eastern populations. Since then, although MG has stabilized in the east to endemic levels, males in at least some populations remain on average redder than they were prior to the epidemic (Hill 2002). In subsequent controlled infection experiments, redder males from unexposed populations showed more rapid clearance of symptoms of MG infection than did yellower males (Hill and Farmer 2005), suggesting possible underlying molecular mechanisms mediating the abilities of more and less ornamented males to respond to and survive infection.

To date, the molecular mechanisms that might connect plumage coloration to disease resistance are poorly understood (Hill and Johnson 2012; Hill 2014). The appropriate response of wild animals challenged by natural pathogens is often unclear due to the complexity of the immune system. In addition, the mechanisms responsible for the modification and deposition of ingested pigments responsible for sexually selected colors are still unknown (Walsh et al. 2012; Pointer et al. 2012). So far connections between mechanisms of carotenoid ornamentation and immune function have not been adequately examined due to 1) "quasi-inexistent" knowledge of the genetics of carotenoid-based coloration (Roulin and Ducrest 2013) and 2) a one-size-fits-all approach to immunity.

Here, we studied the transcriptional mechanisms by which plumage coloration of wild male house finches from a population with no history of infection with MG predicts their ability to respond to infection. MG is a conjunctivitis-causing natural respiratory pathogen of some populations of house finches, but at the time of this study was not known to occur in the southwestern United States (Bonneaud et al. 2011). Finches in eastern United States populations have been under strong selection from the detrimental effects of MG, resulting in low levels of plumage color variation since the initial epidemic of MG (Nolan et al. 1998; Dhondt et al. 2006) and, when exposed to MG in controlled experiments, eastern finches have a lower pathogen load 14 days following infection than do southwestern finches (Bonneaud et al. 2011). Moreover, southwestern finches show down-regulation of immune and immune-associated genes following infection; whereas, eastern finches do not, suggesting that eastern birds do not experience the immunosuppressive effects generally associated with MG infection and have evolved increased resistance (Bonneaud et al. 2011).

Based on these preliminary observations, we predicted that male house finches from unexposed populations with red plumage would be better able to respond to MG infection than males from those same populations with yellow plumage and that this enhanced responsiveness would be associated with patterns of gene expression similar to those found in MG-adapted eastern house finches. To this end, we used a house finch-specific cDNA microarray to test for differences in gene expression between a small sample of red and yellow male house finches following experimental infection with MG. We first asked whether there were significant splenic transcriptional differences between infected and control birds related to ornamental plumage color. We used the results of these expression comparisons as well as previously published results of comparisons between birds from eastern (exposed) and southwestern (unexposed) populations (Bonneaud et al. 2011) to identify a set of candidate genes to study in a larger sample of male finches with red and yellow plumage color. We performed quantitative multiplexed reverse transcription polymerase chain reaction (RT-PCR) on individual birds and analyzed expression levels of 16 candidate genes in relation to infection response and plumage color. Finally, we tested for functional significance of differences in expression of these genes by comparing transcript abundance in infected finches to the severity of their symptoms.

METHODS

Study animals and experimental conditions

Wild male house finches were captured in January 2007 and August 2010. Birds used in this study were captured in south-central and southeastern Arizona, where natural infection with MG had not occurred at the time of the investigation. We chose to study birds from a population without a history of MG infection because eastern populations have low levels of plumage color variation since the initial epidemic of MG (i.e., most male house finches from exposed populations have long wavelength hues; Nolan et al. 1998) and have evolved resistance to MG (Bonneaud et al. 2011). In addition, a previous study that found redder male finches were able to recover more quickly from infection was conducted on birds from an unexposed population in Hawaii (Hill and Farmer 2005). By utilizing birds from populations that have not evolved resistance as a whole, we increased our ability to identify genes that exhibit variation with respect to plumage coloration in response to infection. In 2007, a total of 36 birds were captured in Tempe, Arizona only; in 2010, a total of 67 birds were captured from 3 separate locations in southeastern Arizona (Tempe, Green Valley, and Tucson). Of those, 19 and 37 of the most red and most yellow birds in 2007 and 2010, respectively, were chosen for inclusion in this experiment, whereas the remainder were retained for separate studies (e.g., Bonneaud et al. 2012a; Hill et al. 2013; see Supplementary Table S1 for study sample sizes). We specifically selected birds with the most extreme hues so as to increase our power to detect differences while at the same time reducing the number of animals necessary for inclusion in this terminal study. Birds were transported in cages to Auburn, Alabama by air within 48 h of capture in 2007 and by van within 96 h of capture in 2010 (see Hill et al. 2013 for details of 2010 transport). In both years, finches were subsequently housed at Auburn University in pairs in wire-mesh cages (0.5 m^3) in temperature-controlled rooms (1.5 m × 2.1 m × 2.4 m) with large windows allowing for abundant exposure to natural light. Birds were maintained for 4 and 2 months in 2007 and 2010, respectively, prior to the start of the experiments to allow them to acclimate to captivity in a common environment. We tested for presence of and exposure to MG using PCR of choanal swabs (2007 only; Roberts et al. 2001) and a serum plate agglutination assay (2007 and 2010; Luttrell et al. 1996) both on arrival and at the end of the quarantine period. All birds used in experiments tested negative for MG. All animal protocols and procedures were approved by the Institutional Animal Care and Use Committee of Auburn University (IACUC protocols #2007-1197 and #2010-1762).

We measured reflectance spectra of feathers across the avian visual range (300–700 nm) following standard procedures (Montgomerie 2006) using an Ocean Optics S2000 spectrometer (Dunedin, FL). In 2007, we plucked 15 breast feathers from each bird within 1 week of capture and taped them to a black card for measurement. In 2010, we measured plumage color directly from the breasts of males on the day they arrived in Auburn from Arizona. We quantified hue, the most relevant descriptor of male house finch color (Hill 2002), as the wavelength of 50% reflectance (λR50) located at the midpoint between the maximum and minimum reflectance within the visible spectrum (Pryke et al. 2001). Hues clustered into 2 groups: long wavelength (hereafter called red; $N = 26$, $\bar{x} = 606$ nm ± 1.4 SE), and short wavelength (hereafter called yellow; $N = 30$, $\bar{x} = 568$ nm ± 1.9 SE).

Birds were inoculated with SP4 sterile media (controls) or a cultured MG field isolate collected in January 2007 (infected groups)

Downloaded from http://beheco.oxfordjournals.org/ by guest on May 8, 2015

following standard protocols (Farmer et al. 2002; Bonneaud et al. 2011; BUA #243 and #500). Briefly, birds are infected with 20 μL of 1×10^4 to 1×10^6 color changing units of culture via ocular inoculation. In both years, control and infected birds were maintained under identical conditions but in separate containment rooms. Birds were monitored every 1–3 days for symptoms of conjunctivitis. On the final day of the experiment, all birds were captured and scored for conjunctivitis in each eye using a scale from 0–3. A score of 1 reflected minimal signs of the disease whereas 3 referred to an eye that was completely blind from conjunctival swelling. Left and right eye scores 14 days following infection were then summed to produce a total conjunctival score. Birds were bled and swabbed 14 days postinfection to test for seroconversion and the presence of MG DNA in the choanal cleft (Roberts et al. 2001). All birds were euthanized 14 days postexposure as previous work has shown population-level differences in gene expression in response to MG at this timepoint (Bonneaud et al. 2011, 2012b). Spleen tissues were removed immediately following death and stored in RNAlater (Ambion, Austin, TX) at 4 °C for 24 h and then placed at −80 °C until extractions were conducted.

RNA extractions

Total RNA was extracted from approximately 17 mg of each individual's spleen tissue. RNA was extracted using RNeasy miniprep spin columns (Qiagen, Valencia, CA) followed by genomic DNA digestion using Turbo DNase (Ambion, Austin, TX) according to manufacturers' protocols. We quantified the amount of total RNA using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE) and determined the integrity of the RNA using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). RNA extracts were then stored at −80 °C.

Microarray of house finch cDNA

Construction

Descriptions of house finch cDNA microarray slides are published in detail elsewhere (Bonneaud et al. 2011, 2012a). Briefly, we constructed a cDNA microarray using differentially expressed and control cDNA clones from the subtraction suppression hybridization (SSH) libraries described in Wang et al. (2006). These SSH libraries were specifically enriched for clones that were up- and down-regulated in the spleens of house finches 14 days following experimental inoculation with a 2001 culture of MG (Wang et al. 2006). The microarray included approximately 1000 unique clones, including all 220 clones found to be significantly differentially expressed between healthy and infected house finches in a previous study using macroarrays (Wang et al. 2006), as well as an additional 694 clones that were haphazardly selected from the SSH libraries. Although these clones were present in the SSH libraries, their identities and response to infection were unknown. We also printed 5 house finch housekeeping genes. These genes were generated by PCR amplification of cDNA extracted from house finch spleens using degenerate primers. We quantified purified PCR products on a Nanodrop spectrophotometer. In addition, we printed the PCR products from DNA amplifications of 11 *Escherishia coli* housekeeping genes to serve as external spike-ins (Bonneaud et al. 2011).

Hybridization and visualization

House finch RNA samples and *E. coli* external spike-in housekeeping genes were prepared for cDNA microarray hybridization as described in detail in Bonneaud et al. (2011). Only samples collected in 2007 were used in microarray comparisons. We pooled samples by treatment and plumage color (N = 4 red control birds [$\bar{X}$ = 606 nm]; N = 3 red infected birds [$\bar{X}$ = 603 nm]; N = 2 yellow control birds [$\bar{X}$ = 576 nm]; N = 3 yellow infected birds [$\bar{X}$ = 579 nm]) and hybridized these 4 total pools against a common reference sample, consisting of an aliquot of all samples, onto our custom microarray. Following hybridization, microarray slides were scanned for visualization of fluorescent probes using an Axon 4000A microarray scanner (Molecular Devices, Sunnyvale, CA).

Multiplexed quantitative RT-PCR of candidate genes

We measured transcriptional changes in the spleen of birds collected in 2007 and in 2010 (N = 19 control; N = 38 infected) across 18 candidate genes and 2 housekeeping (control) genes using multiplexed quantitative real-time amplifications (details of assay and accession nos. are provided in Balenger et al. 2011). Genes were short-listed as candidates if they were 1) identified as significantly differentially expressed between infected red or yellow birds relative to controls using microarrays (this study) and between infected eastern and southwestern birds using microarrays (n = 17, Bonneaud et al. 2011), 2) identified as significantly differentially expressed only between infected red or yellow birds relative to controls using microarrays (n = 1, this study), and 3) identified as significantly differentially expressed only between infected eastern and southwestern birds using microarrays (n = 34, Bonneaud et al. 2011). From these 53 genes, we selected a subset of 30 by focusing on those whose clones were long enough to design primers and that are known to function in immune, metabolic, and stress responses (Balenger et al. 2011). The final 18 genes meet all of the above criteria, plus we were able to design RT-PCR primers that were gene specific in the context of a single highly multiplexed reaction (Balenger et al. 2011).

We generated a standard curve using a 2-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 from control and treatment birds. We used Beckman Coulter protocols for each reverse transcription reaction using GeXP Start kit reagents unless otherwise noted: 4.0 μL of RT buffer (5×), 2.0 μL of a pool of attenuated reverse primers (10×), 1.0 μL of reverse transcriptase, 3.0 μL of DNase-free water, 5.0 μL of 0.625 ng/μL external spike-in control RNA (*kanR*) and 5.0 μL of 5 ng/μL sample mRNA. The concentration of each reverse primer varied from 0.01 to 5 μM (see Balenger et al. 2011, Table 1 for reverse primer dilutions). 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 consisted of 4.0 μL of PCR buffer (5×), 4.0 μL of MgCl2 (ABgene, Rockford, IL), 2.0 μL of a 10× pool of forward primers all at 2 μM concentration, 0.7 μL of Taq polymerase (ABgene), and 9.3 μL of cDNA from the RT reaction. Cycling parameters were 95 °C for 10 min, followed by 35 cycles for 94 °C for 30 s, 55 °C for 30 s, and 70 °C for 1 min, and a final extension step of 70 °C for 10 min. Forward and reverse primer sequences are given in Table 1 of Balenger et al. (2011).

Of the 18 candidate genes targeted by this multiplex, all were previously identified as differentially expressed between infected house finches from eastern and southwestern populations (Bonneaud et al. 2011). In addition, 7 of these were also identified in this study as significantly differentially expressed in the spleens of yellow infected birds relative to controls based on microarray comparisons (see Results); that is, expression of these 7 genes also appeared to be different between infected yellow and infected red

Downloaded from http://beheco.oxfordjournals.org/ by guest on May 8, 2015

Table 1

Summary of house finch clones with known identity (i.e., shared sequence homology) that were found to be significantly differentially expressed using microarray hybridization

HGNC name	HGNC identifier	GO category	Identity	e-value	Accession no.	# of clones	Fold change	FDR adjusted *P*-value	Plumage color
hCG40889, CFH	*cfh*	Immune	[a]227/257	8E−78	XM_002192303.1	1	2.1	0.032	Yellow
hCG40889, CFH	*cfh*	Immune	[a]227/257	8E−78	XM_002192303.1	1	3.4	0.046	Red
MHC class II-associated invariant chain Ii	*mhcIi*	Immune	[a]565/665	1E−174	DQ215319.1	17	−7.1	0.016	Yellow
Lectin galactoside-binding soluble 2 protein	*lgals2*	Immune	[a]371/392	4E−170	XM_002196008.1	1	−4.2	0.020	Yellow
Erythrocyte membrane protein band 4.1-like 2	*epb41l2*	Immune, Cytoskeleton	[a]593/612	0	XM_002192813.1	1	−3.9	0.016	Yellow
T cell receptor β chain	*tcrb*	Immune	[c]284/347	1E−71	AF068228.1	1	−3.2	0.016	Yellow
Spermidine/spermine N1-acetyltransferase	*sat1*	Polyamine catabolism	[a]197/201	7E−93	XM_002195920.1	1	−2.3	0.046	Yellow
Prosaposin	*psap*	Sphingolipid metabolism	[a]262/311	3E−72	DQ214627.1	1	−2.2	0.043	Yellow
Heat shock protein 70B	*hsp70*	Stress	[a]230/236	6E−109	XM_002195700.1	1	−2.2	0.039	Yellow
Heat shock protein 90a	*hsp90*	Stress	[a]244/253	9E−112	XM_002200572.1	3	2.1	0.039	Yellow
Translation initiation factor EIF4G2	*eif4g2*	Translation	[a]637/656	0	XM_002197226.1	2	−4.4	0.017	Yellow
Ribosomal protein S15	*rps15*	Translation	[a]126/132	5E−50	DQ213656.1	1	−2.1	0.029	Yellow
Eukaryotic translation initiation factor 4E	*tif*	Translation	[a]818/831	0	DQ213184.1	1	2.2	0.029	Yellow
Actin, gamma 2 propeptide	*actg2*	Cytoskeleton	[a]155/157	1E−71	XM_002190324.1	1	−3.7	0.016	Yellow
Destrin	*dstn*	Cytoskeleton	[b]523/631	2E−143	NM_205528.1	2	−2.8	0.036	Yellow
Myosin, light chain kinase	*mylk*	Muscle contraction	[b]237/253	1E−100	AF045285.1	1	−4.6	0.016	Yellow
Secretory complex 61 γ subunit	*sec61γ*	Transport	[a]192/197	6E−89	XM_002198448.1	1	−6.1	0.016	Yellow
Ubiquitin C	*ubc*	Proteolysis	[a]639/691	0	DQ216247.1	4	−4.2	0.020	Yellow
Pleckstrin homology domain	*phip*	Signal transduction	[a]346/359	1E−165	XM_002189748.1	1	3.0	0.017	Yellow

Provided are HUGO Gene Nomenclature Committee (HGNC) identifiers, vertebrate homolog as identified by BLAST, and gene ontology (GO) category; many genes were implicated in several biological processes and when that occurred, we list the processes associated with immune functioning or stress response. We also provide the identity, e-value and accession number of the sequence sharing the greatest homology in GenBank, which was used to determine putative function for differentially expressed clones. The number of clones that shared significant homology to the accession number, the largest fold change of those clones (if >1) in expression of infected finches relative to control finches, *P*-values adjusted for false discovery rate, and the plumage color of the infected birds differentially expressing the gene in comparison to controls are also provided.

Species identified with highest sequence homology (and its common name):

[a]*Taeniopygia guttata* (zebra finch).

[b]*Gallus gallus* (chicken).

[c]*Anas platyrhyncos* (mallard duck).

Downloaded from http://beheco.oxfordjournals.org/ by guest on May 8, 2015

birds based on qualitative comparisons of plumage and microarray results. Standard curve values and raw sample data for each gene was normalized to a transcript of known quantity spiked into each reaction (*kan*®, GenomeLab GeXP Start Kit, Beckman Coulter). Sample data were then interpolated into the standard curve and normalized to a house finch housekeeping gene (actin related protein 2/3) contained in the same multiplex.

Data analyses

Microarray

All analyses of microarray data were performed using R software (http://www.r-project.org). Microarray generated expression data were normalized with a background adjustment using the normexp method, Lowess regression (2-dimensional method) for data smoothing, and scale normalization to control for variation between slides. We fit 2 generalized linear models to normalized $\log_2$ transformed signal ratios (sample vs. reference), with treatment as a factor. The first model compared both pools of control birds with the single pool of infected red birds; the second model compared the same control pools with the single pool of infected yellow birds. It was necessary to use both control pools for each analysis to generate *P*-vales. *P*-values were adjusted for multiple testing using the false discovery rate (FDR) correction with $\alpha = 0.05$ (Benjamini and Hochberg 1995). We sequenced clones found to be significantly differentially expressed between groups as described previously (Bonneaud et al. 2011). These sequences were deposited in Genbank (accession numbers are provided as Supplementary Material). Sequences generating a Basic Local Alignment Search Tool (BLAST) hit with an e-value < 1×10^{-20} with more than 100 nucleotides were assigned to their vertebrate homolog. Gene ontology category and function were determined using Harvester (http://harvester.fzk.de/harvester/).

qRT-PCR

All statistical analyses of qRT-PCR data were performed using SAS version 9.3 (SAS Institute, Cary, NC). In 2007, we collected birds from a single site, and in 2010 we collected birds from that same site and 2 other locations (see Study Animals and Experimental Conditions). Among birds collected in 2010, we determined that site of capture did not significantly affect expression of any candidate genes (Anovas: df = 2, all $P > 0.1$) or conjunctival swelling (multinomial logistic regression: $\chi^2 = 3.52$, df = 2, $P = 0.17$), and so was not considered in final analyses. Examination of 2007 and 2010 qRT-PCR data using Anovas showed that year had a significant effect on expression of all candidate genes ($N = 37$–56, df = 1, all $P < 0.03$). Expression data for each gene was therefore standardized by year to a mean = 0 and a standard deviation (SD) = 1. Standardized gene expression data was not significantly different across years ($N = 37$–56, df = 1, all $P > 0.80$).

We used Pearson correlations to explore relationships between gene expression in the spleen of the 18 house finch candidate genes and to determine the potential for correlated responses. Sample sizes vary between genes due to lack of amplification of 1 or more loci in some reactions. We found at least 1 significant bivariate relationship for each of the 18 candidate genes amplified using qRT-PCR, for a total of 103 out of 153 (67%) correlations showing significance (Supplementary Table S2). Due to this extensive covariation in expression and the large number of comparisons required by investigating 16 candidate genes, we chose to perform a principal components analysis (PCA) on standardized gene expression values. As a result, it was necessary to remove 2 genes, *dstn* and *map*, from subsequent analysis because these were not measured in 2007 samples and would have reduced the overall number of samples in the study substantially. Furthermore, at least 1 gene was not successfully amplified for 8 birds ($N = 1$ red control; $N = 3$ yellow controls; $N = 2$ red infected; $N = 2$ yellow infected) and those individuals were removed from further analyses involving gene expression. We therefore ultimately extracted principal component scores for 16 candidate genes across 48 individuals (17 from 2007 and 31 from 2010), retaining only those components with eigenvalues >1. We used the resulting scores in subsequent analyses of gene expression. Analyses of expression of individual genes with respect to treatment and plumage color are qualitatively similar to those reported for the 3 PC scores (Supplementary Figure S1).

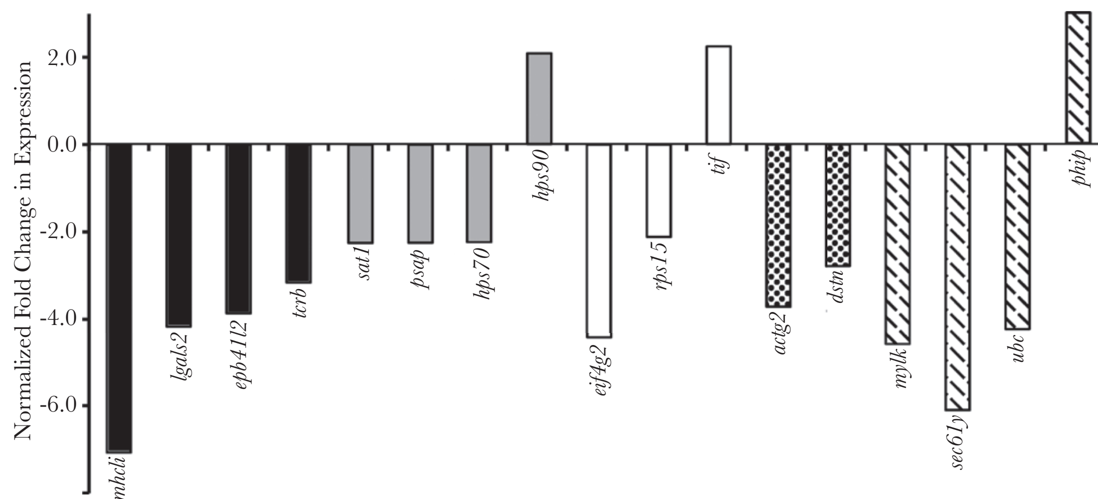

Figure 1

Statistically significant changes in expression levels as measured using a cDNA microarray. Comparison shown is between infected yellow males relative to uninfected control birds (both red and yellow) for genes of known function. When more than 1 clone was detected for a gene ($n = 5$), we show the largest fold change for that gene. Bar colors represent general functional category determined using Harvester (black – immune; gray—redox metabolism/stress; white—translation; dotted—cytoskeleton; dashed lines—other). Not shown: *hCG40889* (CFH) was significantly differentially expressed between infected and control birds for both plumage color groups (fold-change in expression relative to controls: yellow infected = 3.4×; red infected = 2.1×) and therefore considered unlikely as a candidate for differential expression directly between plumage color groups.

Downloaded from http://beheco.oxfordjournals.org/ by guest on May 8, 2015

We performed generalized linear models to test whether principal components of gene expression were predicted by treatment group (control vs. infected) and plumage color (red vs. yellow). We tested whether plumage color alone predicted the severity of disease symptoms (eye swelling) on day 14 using an ordinal regression model with cumulative logit links. Generalized linear models were used to evaluate whether the eye swelling of infected birds was predicted by plumage color and gene expression. We performed post hoc analyses on the individual genes comprising any principal components in which the factor of primary interest, the interaction of treatment and color, was significant. Significance of generalized linear models was determined using type III sums of squares and regression models were evaluated based on type 3 Wald χ^2 values.

RESULTS

Microarray of house finch cDNA

Microarray comparisons of pooled samples collected in 2007 detected 82 (11%) significantly differentially expressed clones between control and infected birds. All 82 were significantly differentially expressed between infected yellow birds and controls, whereas 7 out of these 82 were also significantly differentially expressed between infected red birds and controls. Identities of many of the clones were redundant and after sequencing were determined to correspond to the same gene; using BLAST we could provide identities for 18 individual genes from 41 of the clones (see Table 1 for additional details regarding gene identities). Most of the 18 differentially expressed genes were identified from only a single clone (n = 13), but 5 were identified by 2 or more clones (*tif* and *destrin*, n = 2; *hsp90*, n = 3, *ubc*, n = 4; and *mhcIi*, n = 17). When more than 1 clone blasted to the same gene, the direction of change was always in the same direction (Table 1). The remaining clones either blasted to mRNA from Zebra Finch (*Taenyopygia guttata*) of unidentified function (n = 23), or did not yield any hits (n = 18), and thus their functions are unknown. Of genes with identified functions, only the gene *hCG40889*, which encodes complement factor H (CFH), was differentially expressed by both color groups relative to controls. CFH expression increased 3.4-fold in infected red birds compared with controls, and 2.1-fold in infected yellow birds. Of the remaining, 17 differentially expressed genes of known function, of particular interest are those known to have primary and/or auxiliary functions in immune processes (N = 5), stress response (N = 2), and redox metabolism (N = 2; Figure 1; Table 1). None of the genes identified are known to directly function in dietary carotenoid conversion or deposition.

Quantitative RT-PCR of house finch RNA

PCA of expression levels of 16 genes measured in birds from 2007 and 2010 yielded 3 components with eigenvalues >1, which combined described 80% of the total variation in gene expression (Table 2). The first principal component (PC1) included high (>|0.6|), positive factor loadings for 11 out of 16 candidate genes (Table 2), including all 5 genes tested with primary immune function (*ig4a, igJ, mhcIi, ptms*, and *tcr*). The second principal component (PC2) included high, positive factor loadings for 3 candidate genes: *hsp90, nabp*, and *ubc*. PC3 had high, positive factor loadings for *nadh* and *tif*.

Treatment showed a marginally significant effect on PC1 gene expression, with decreased expression of these 11 genes tending to be associated with MG infection (p = 0.06, Figure 2; Table 3). PC1 was not significantly predicted by color or the interaction of treatment and color (Figure 2; Table 3). Plumage color on its own

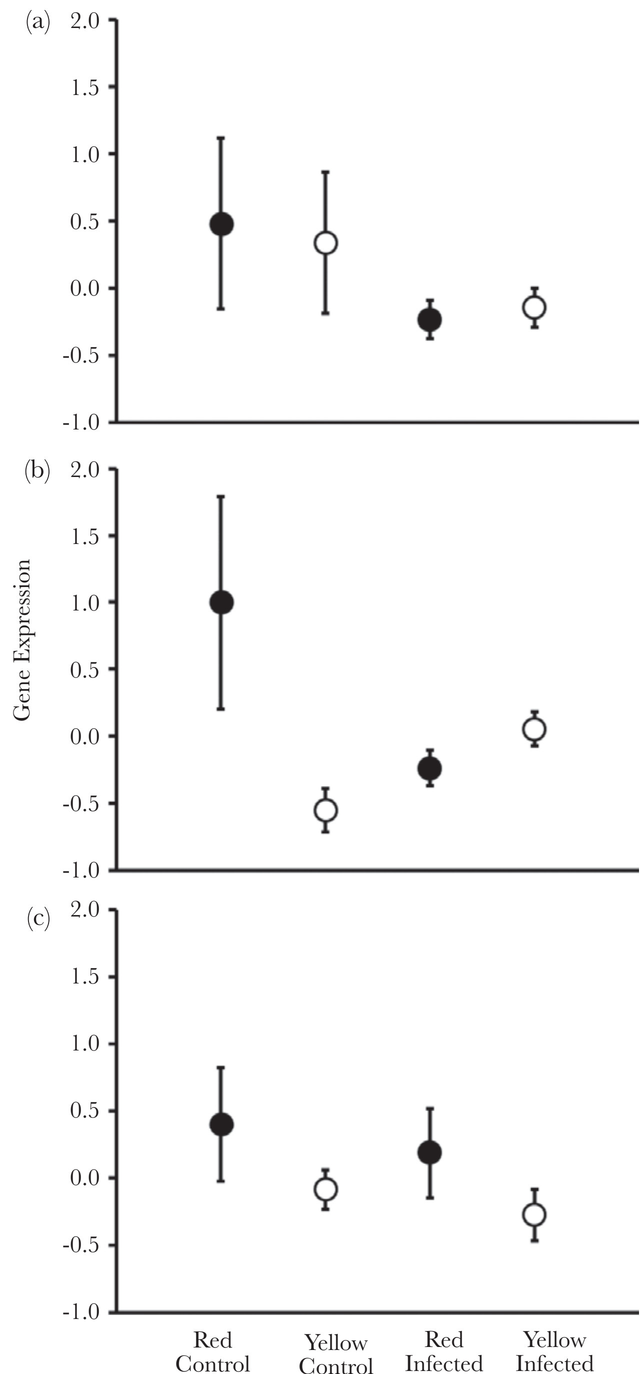

Figure 2
Expression of candidate genes (as measured by qRT-PCR) of red (filled circles) and yellow (open circles) male house finches 14 days after they were inoculated with sterile media ("Control") or infected with the pathogenic bacterium MG ("Infected"). Gene expression scores were derived from a PCA: (a) high PC1 values represent high expression of *ick, ig4a, igJ, lcp, mhcIi, psap, ptms, rhoa, sec61γ, tcr*, and *txn*; (b) high PC2 values represent high expression of *hsp90, nabp*, and *ubc*; and (c) high PC3 values represent high expression of *nadh and tif*. Shown are means ± 1 SE.

and the interaction of treatment and color were both significant predictors of PC2 (Figure 2; Table 3). That is to say, irrespective of treatment, red birds expressed significantly higher levels of *hsp90*, *nabp*, and *ubc* than did yellow birds, although this appears to be

Downloaded from http://beheco.oxfordjournals.org/ by guest on May 8, 2015

largely driven by the extremely high levels of expression in control red birds compared with all other experimental groups (Figure 2). We explored significance of the interaction factor with post hoc one-way Anovas and found that infection with MG resulted in an increase in PC2 among yellow birds ($F = 7.71$, $P = 0.01$, $r^2 = 0.24$) and a decrease in PC2 among red birds ($F = 4.82$, $P = 0.04$, $r^2 = 0.19$). Among control birds, there was marginally significant effect of plumage color, such that yellow control birds exhibited decreased expression of PC2 when compared with red control birds ($F = 4.15$, $P = 0.06$, $r^2 = 0.24$). Following infection with MG for 14 days, however, expression of these genes was not significantly different between birds in the 2 plumage color groups ($F = 2.55$, $p = 0.12$, $r^2 = 0.08$). Neither treatment, color, nor their interaction was significant predictors of PC3 (Figure 2; Table 3). Full models were also run without birds included in the microarray hybridizations and provided similar results of the key findings: treatment was a significant predictor of PC1 gene expression ($P = 0.02$), whereas the interaction of treatment and color was a highly significant predictor of PC2 gene expression ($P = 0.008$) but color on its own was no longer significant ($P = 0.11$; Supplementary Figure S2 and Table S3).

Post hoc analysis of individual genes that loaded primarily on PC2 showed highly significant effects of treatment and color on expression of *ubc* and the interaction of treatment and color on both *ubc* and *hsp90* (Supplementary Table S4). None of these factors were significant predictors of *nabp* expression on its own (Supplementary Table S4).

Severity of infection

Conjunctival swelling of infected birds did not differ significantly between the 2 plumage color groups 14 days after initial inoculation ($X^2 = 1.24$, df = 1, $P = 0.27$). Furthermore, conjunctival swelling was not significantly predicted by models examining plumage color group and PC1 or PC3 (Figure 3; Table 4). However, when considered in relation to PC2 and plumage color, swelling was greater in yellow birds than in red birds, greater in birds with lower PC2 scores, and exhibited a significant interaction effect in response to color and PC2 expression (Figure 3; Table 4). Post hoc analyses found no significant relationship between gene expression of infected yellow males ($n = 19$) and eye swelling (*hsp90*: $X^2 = 0.93$, df = 1, $P = 0.33$; *nabp*: $X^2 = 0.00$, df = 1, $p = 0.97$; *ubc*: $X^2 = 0.00$, df = 1, $p = 0.99$), but did identify a significant relationship between *ubc* expression of infected red birds ($n = 18$) and eye swelling (*hsp90*: $X^2 = 1.63$, df = 1, $P = 0.20$; *nabp*: $X^2 = 0.07$, df = 1, $P = 0.79$; *ubc*: $X^2 = 4.14$, df = 1, $P = 0.04$).

DISCUSSION

Multiple models of sexual selection propose that the quality or condition of an ornamental trait used in mate choice signifies the

Table 2

Results of PCA on expression of candidate genes in spleen tissue of 48 house finches

			Factor loading			
HGNC name	HGNC identifier	GO category	PC1	PC2	PC3	Where identified
Serine/threonine kinase	*ick*	Signal transduction	**0.82**	0.16	−0.01	1
Immunoglobulin 4α	*ig4α*	Immune	**0.83**	0.25	−0.09	1
Immunoglobulin J	*igJ*	Immune	**0.84**	−0.22	0.17	1
Lymphocyte cytosolic protein	*lcp*	Cytoskeleton (Immune)	**0.93**	0.15	0.20	1, 2
MHC class II associated invariant chain Ii	*mhcIi*	Immune	**0.91**	0.15	0.09	1, 2, 3
Prosaposin	*psap*	Metabolism	**0.80**	0.12	−0.01	1, 3
Parathymosin	*ptms*	Immune	**0.64**	0.40	0.48	1
RhoA GTPase	*rhoa*	Oxidative burst	**0.80**	0.45	0.30	1
Secretory complex 61 γ subunit	*sec61γ*	Transport	**0.87**	0.18	0.26	1, 3
T cell receptor β chain	*tcrb*	Immune	**0.75**	−0.01	−0.01	1, 3
Thioredoxin	*txn*	Redox metabolism (Immune)	**0.89**	0.15	0.20	1
Heat shock protein 90a	*hsp90*	Stress	−0.04	**0.97**	−0.01	1, 2, 3
Nucleic acid binding protein	*nabp*	Nucleic acid binding	0.25	**0.74**	0.55	1
Ubiquitin C	*ubc*	Proteolysis (Immune)	0.35	**0.67**	0.49	1, 2, 3
Nicotinamide adenine dinucleotide	*nadh*	Redox metabolism	−0.10	0.06	**0.84**	1
Eukaryotic translation initiation factor 4E (eIF4E)	*tif*	Translation	0.30	0.19	**0.74**	1, 3
Eigenvalue			9.24	2.50	1.14	
Proportion of variance explained			0.58	0.16	0.07	

Candidate genes were measured using qRT-PCR. Each gene is described using its HGNC name and identifier and its primary gene ontogeny (GO) category. Genes having auxiliary immune function are noted in parentheses. Shown are factor loadings (correlation between components and original variables) for each of the 16 genes for the first 3 PCs (PC1, PC2, and PC3). Factor loadings ≥ |0.60| are bolded. Also provided are citations for where this gene has been identified as differentially expressed in the spleen of house finches in relation to infection with MG. At the bottom of the table are eigenvalues and the proportion of the variance in gene expression explained for each of the PCs.

1 Bonneaud et al. (2011)—identified this gene (using microarrays) as significantly differentially expressed between infected birds from a population coevolving with MG and infected birds from a population with no prior history with MG;

2 Wang et al. (2006)—identified this gene (using macroarrays) as significantly differentially expressed between birds infected with MG and birds not infected; and

3 this study—identified this gene (using microarrays) as significantly differentially expressed between yellow birds infected with MG and birds not infected, but not significantly differentially expressed between red birds infected with MG and birds not infected.

Downloaded from http://beheco.oxfordjournals.org/ by guest on May 8, 2015

bearer's ability to respond to parasites and pathogens (Hamilton and Zuk 1982; Borgia 1979; Folstad and Karter 1992; Able 1996). Here, we examined whether male house finch plumage color reflects ability to respond to the bacterial pathogen MG at both the level of symptom severity and gene expression in a primary immune tissue, the spleen. We found no difference in symptom severity (i.e., eye swelling) 14 days after inoculation when directly comparing red versus yellow infected males. Using microarrays to measure gene expression, however, we found that pooled spleen samples from red and yellow males qualitatively differed in their response to experimental infection. Red males infected with MG showed very few differences in gene expression when compared with controls. In contrast, yellow males responded to infection by altering the expression of a greater number of genes, including the down-regulation of genes related to immune response (*mhcIi*, *epb41l2*, *lgals2*, *psap*, and *tcrb*) and cellular protection (*hsp70*), and the up-regulation of a stress-response gene (*hsp90*). Based on these observations of birds obtained from a population with no prior experience with the MG bacterial pathogen, house finch plumage color appears capable of indicating an individual's ability to express genes associated with responding to MG infection. Of note, all but one of the genes identified as differentially expressed (*hsp70*) in this experiment were also identified as contributing to population-level differences in resistance by house finches infected with MG (Bonneaud et al. 2011).

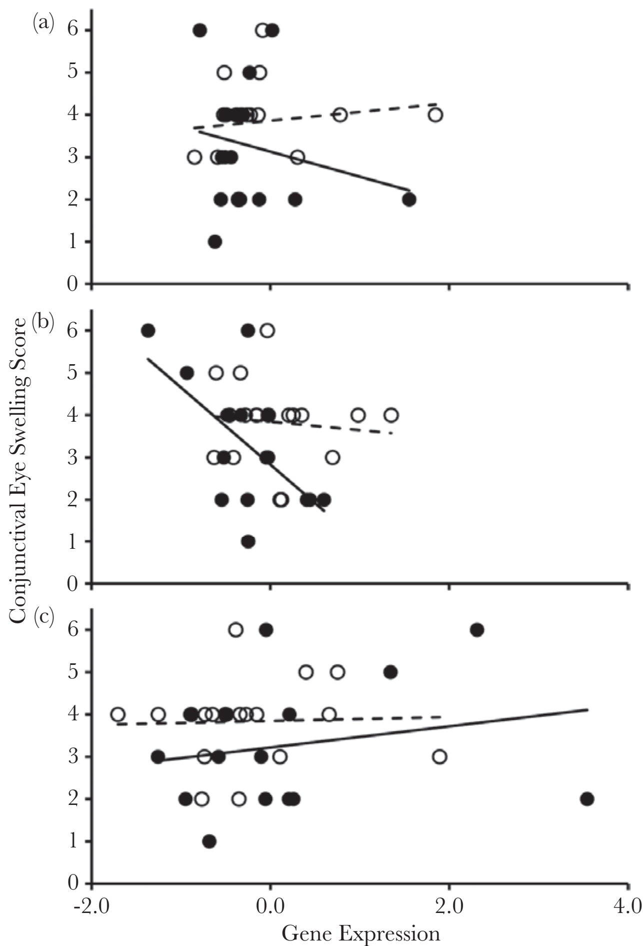

Figure 3
PC scores of expression of 16 candidate genes measured using qRT-PCR in house finch spleen tissue. Conjunctival swelling around the eye in red finches infected with MG for 14 days (filled circles) and yellow finches infected with MG for 14 days (open circles) with respect to (a) PC1, (b) PC2, and (c) PC3 scores. Linear regression lines are plotted for red (solid) and yellow (dashed) males.

Mycoplasmas, including MG, can modulate their host's immune system to evade detection, and MG infections are associated with evidence of immunosuppression (Mühlradt 2002; Ganapathy and Bradbury 2003; Mohammed et al. 2007). The patterns of gene expression we identified here using microarrays suggest a qualitatively poorer response to MG infection by drably colored yellow males relative to red males. In particular, genes related to immune-response were down-regulated in yellow birds but not red birds, suggesting that the immune systems of yellow males were being suppressed as a result of MG infection. These gene expression patterns were qualitatively similar to patterns observed in relation to population of origin in earlier studies, with Arizona (no prior evolutionary history with MG) and Alabama (coevolving with MG for approximately 14 years) birds displaying gene expression responses reminiscent of yellow and red birds, respectively (Bonneaud et al. 2011).

Subsequent quantitative amplification of 16 candidate genes enabled us to characterize variation in gene expression across individuals associated with both plumage color and severity of symptoms 14 days following infection. PCA of expression of these genes revealed 3 general patterns of expression: genes that decrease expression in response to infection regardless of plumage color (PC1, n = 11 genes), genes that respond to infection by decreasing expression in red birds and increasing expression in yellow birds (PC2, n = 3 genes), and genes that may be more highly expressed in red birds than in yellow birds regardless of infection status (PC3, n = 2 genes), although these were not significantly different. The pattern shown by PC1 genes supports findings in Bonneaud et al. (2011) in which all 16 of the genes investigated here were found to be significantly differentially expressed by finches from Arizona in response to infection with MG (Bonneaud et al. 2011). Such

Downloaded from http://beheco.oxfordjournals.org/ by guest on May 8, 2015

Table 3
Results of generalized linear models testing whether treatment group (control vs. infected with MG), plumage color (red or yellow), and/or their interaction predict the expression of candidate genes in the spleens of house finches (n = 48)

	Treatment			Color			Treatment x color			Model
	F	η^2	p	F	η^2	p	F	η^2	p	r^2
PC1	3.73	0.08	**0.06**	0.01	0.00	0.93	0.13	0.00	0.72	0.08
PC2	1.22	0.02	0.27	4.86	0.09	**0.03**	10.46	0.19	**0.002**	0.22
PC3	0.42	0.01	0.52	2.31	0.05	0.14	0.00	0.00	0.97	0.07

PC refers to principal component factor. Effect sizes are given for each factor in the model (η^2) as well as for the complete model (r^2). p-values < 0.05 are given in bold.

Table 4

Results of generalized linear models testing whether gene expression, plumage color (red or yellow), and/or their interaction predict conjunctival swelling of house finches infected with MG (n = 33)

	Expression			Color			Expression x Color			Model
	F	η^2	p	F	η^2	p	F	η^2	p	r^2
PC1	0.23	0.00	0.63	2.38	0.07	0.13	1.00	0.03	0.32	0.09
PC2	6.75	0.17	**0.01**	5.82	0.14	**0.02**	4.36	0.11	**0.05**	0.29
PC3	0.41	0.01	0.53	1.82	0.06	0.19	0.19	0.01	0.66	0.08

PC refers to principal component factor describing gene expression. Effect sizes are given for each factor in the model (η^2) as well as for the complete model (r^2). p-values < 0.05 are given in bold.

concordance between the 2 studies supports the robustness of the same microarray and qRT-PCR platforms used in the current study.

Although plumage color on its own did not predict the severity of eye swelling experienced by a male house finch 14 days following infection with MG, a male's color was associated with disease severity when gene expression was taken into account. Specifically, in an examination of the effect of PC2 (*hsp90*, *nabp*, and *ubc*) expression and color on disease severity we found that correlated expression of these 3 genes, plumage color group, and the interaction of gene expression and color were all important predictors of eye swelling. Surprisingly, birds with red plumage that expressed lower levels of PC2 genes were more likely to display severe clinical symptoms, whereas birds with yellow plumage did not display a relationship between expression of these genes and their symptoms.

Of the 16 candidate genes analyzed with qRT-PCR data, only a subset of 6 were identified in the current study using microarrays—5 were significantly downregulated and 1 was significantly upregulated in yellow infected birds compared with controls using microarrays. Two of those 6 genes (*hsp90* and *ubc*) had high, positive loading on the second axis of the PCA, which responded to plumage color and infection when measured with qRT-PCR. Of the 4 remaining genes identified in this study by microarrays and subsequently examined with qRT-PCR, 2 have a primary immune function (*mhcIi*, *tcr*) and loaded onto PC1, whereas the other 2 (*nadh* and *tif*) are involved in redox metabolism and translation, respectively. These genes both loaded onto PC3.

Post hoc analyses of PC2 genes (Supplementary Figure S1) identified interesting patterns of expression by *hsp90* and *ubc*. HSP90 concentrations typically dramatically increase in response to thermal stress, but heat shock proteins also respond to environmental stressors like exposure to toxins, hypoxia, and infection (Csermely et al. 1998; Young et al. 2001; Pockley et al. 2008; Sorensen 2010). HSP90 is also part of the ubiquitination pathway of protein degradation and is crucial for the maintenance of proteasomal stability. It plays an important role in the trafficking of proteins toward the proteasome after which the degraded peptide products can be used as antigens by MHC class I molecules through the cross-presentation pathway (Udono et al. 2009), suggesting indirect immune function. Here, we found a decrease in *hsp90* expression in red birds and an increase in *hsp90* expression in yellow birds in response to infection, which supports the hypothesis that yellow birds are suffering a greater amount of stress in response to infection than are red birds. What is particularly intriguing about this result, however, is that red control birds and yellow infected birds expressed equivalent amounts of *hsp90* in this experiment. Assuming that *hsp90* expression can be used as a proxy for stress would suggest that red birds without MG and yellow birds with MG are essentially as stressed as one another. One possibility supporting this would be if red control birds suffer greater captivity stress than yellow control birds. Hill et al. (2013) recently examined the effect of cage stress on house finches, measuring protein levels of 3 heat shock proteins. Although HSP90 did not significantly increase in response to cage stress (Hill et al. 2013), the authors did not discriminate between males based on plumage color. If captivity is unduly stressful on red birds as a whole, we would expect infected red birds to have similarly high expression of *hsp90*, but they do not. However, the current study can not disentangle the possibility of an interaction between color, infection, and captivity stress. Assuming there is no differential effect of captivity stress on the groups in this study, it is possible that the increased basal *hsp90* expression in red birds is in fact adaptive. Blind cavefish (*Astyanax mexicanus*) express higher basal levels of *hsp90* than their surface counterparts, and it was recently suggested that this may be advantageous for cavefish by providing them with greater baseline stress resistance (Rohner et al. 2013). At this point, however, the high levels of expression of *hsp90* in control red house finches requires further study.

ubc is involved in the endoplasmic reticulum–associated protein degradation pathway, but can also function in the initiation of the immune response (Biederer et al. 1997; Boyer and Lemichez 2004; Arnason et al. 2005). Similar to *hsp90*, *ubc* was highly expressed in control red birds, whereas control yellow birds and infected birds of both color groups expressed similarly low levels of this gene (Supplementary Figure S1). The decrease in expression by red birds when infected reveals that expression of this gene is plastic in males with sexually preferred red plumage, whereas expression in yellow birds does not respond to infection with MG. Post hoc analysis of disease symptoms identified a similar pattern; levels of *ubc* among infected red birds significantly decreased as eye symptoms increased while this relationship was not detected for infected yellow birds. Plasticity in expression of *ubc* may be important to the ability to cope with or recover from novel stressors, including pathogens. Such plasticity, or lack thereof, may be genetic and/or environmentally controlled. Due to the multiple factors influencing plumage color in wild male house finches, *ubc* seems an excellent candidate for sequencing of regulatory regions of birds with more and less attractive feather color as well as examination of its expression with respect to environmental factors likely to vary between red and yellow males, like carotenoid content in diet.

Prior studies have shown that infection by coccidial, viral, and bacterial pathogens during molt caused male house finches to grow drabber, more yellow feathers (Thompson et al. 1997; Brawner et al. 2000; Hill et al. 2004). Moreover, in a previous experimental infection, the plumage color of male house finches predicted the capacity of individuals to recover from MG infection as measured by the reduction in eye swelling between 6 and 8 weeks after inoculation (Hill and Farmer 2005). The distinct patterns of gene expression by red and yellow house finches that

Downloaded from http://beheco.oxfordjournals.org/ by guest on May 8, 2015

we observed in the current study provide a short list of genes whose expression is related to both ornamental color and disease response. This is crucial information for future studies aiming to identify the mechanism through which the color of feathers is capable of signaling the ability of males to resist or recover from MG infection.

The pattern of gene expression exhibited by house finches with red feathers and the association between redness, gene expression, and an individual's ability to respond to infection are consistent with the hypothesis that red plumage coloration serves as an indicator of the functionality of basic cellular systems from which aspects of performance such as immune defense emerge (Hill 2011, 2014). It was recently proposed that the ketolation of red carotenoid pigments, such as those that determine redness in the plumage of house finches, is intimately linked to the vitamin A and redox state of an individual (Hill and Johnson 2012). By this hypothesis, the same cellular mechanisms that create an environment that is conducive to production of red coloration will also enable greater responsiveness of vital systems such as immune defense. The identification of gene products that are differentially expressed in red and yellow finches is an important step in understanding the mechanisms that link quality of ornamentation to disease response and recovery.

SUPPLEMENTARY MATERIAL

Supplementary material can be found at http://www.beheco.oxfordjournals.org/

FUNDING

This work was supported by funds from the AU-OVPR, the AU-CECST and NSF grant IOS-0923600 (GEH and SVE), the Society of Integrative & Comparative Biology, the American Ornithologists' Union, and the Animal Behavior Society (SLB). SLB was supported by a grant from the Alabama EPSCoR; CB by Harvard University, a Marie Curie Reintegration Grant (FP7-PEOPLE-IRG-2008, #239257) and CNRS.

We thank F. F. Bartol and K. Fielman for technical advice and members of the Hood and Hill labs for help with dissections. We also thank M. Staley for assistance with inoculations, E. Brennan for assistance with RNA extractions, M. Rios-Kessler for bird care, A. Surmacki for help quantifying color of feathers, K. McGraw and R. Ligon for logistical support in Arizona, and J. Zhang for assistance with analysis of microarray data. Thanks also to M. Zuk and E. Snell-Rood for thoughtful discussions of the project.

Handling editor: Wolfgang Forstmeier

REFERENCES

Able DJ. 1996. The contagion indicator hypothesis for parasite mediated sexual selection. PNAS. 93:2229–2233.

Alonso-Alvarez C, Perez-Rodriguez L, Mateo R, Chastel O, Vinuela J. 2008. The oxidation handicap hypothesis and the carotenoid allocation trade-off. J Evol Biol. 21:1789–1797.

Andersson M. 1994. Sexual selection. Princeton (NJ): Princeton University Press.

Arnason TG, Pisclevich MG, Dash MD, Davies GF, Harkness TA. 2005. Novel interaction between Apc5p and Rsp5p in an intracellular signaling pathway in *Saccharomyces cerevisiae*. Euk Cell. 4:134–46

Balenger SL, McClure CJW, Hill GE. 2011. Primer design and transcript quantification of a highly multiplexed rt-PCR for a non-model avian species, *Carpodacus mexicanus*. Mol Ecol Res. 12:116–122.

Balenger SL, Zuk M. 2014. Testing the Hamilton–Zuk hypothesis: past, present and future. Integr Comp Biol. doi:10.1093/icb/icu059.

Benjamini Y, Hochberg Y. 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc Ser B (Method). 57:289–300.

Biederer T, Volkwein C, Sommer T. 1997. Role of Cue1p in ubiquitination and degradation at the ER surface. Science. 278:1806–1809.

Blount JD, Metcalfe NB, Birkhead TR, Surai PF. 2003. Carotenoid modulation of immune function and sexual attractiveness in zebra finches. Science. 300:125–127.

Bonneaud C, Balenger SL, Russell AF, Zhang J, Hill GE, Edwards SV. 2011. Rapidly evolving disease resistance manifest through functional changes in gene expression in a wild bird. PNAS. 108:7866–7871.

Bonneaud C, Balenger SL, Zhang J, Edwards SV, Hill GE. 2012a. Innate immunity and the evolution of resistance to an emerging infectious disease in a wild bird. Mol Ecol. 21:2628–2639.

Bonneaud C, Balenger SL, Hill GE, Russell AF. 2012b. Experimental evidence for distinct costs of pathogenesis and immunity against a natural pathogen in a wild bird. Mol Ecol. 21:4787–4796.

Borgia G. 1979 Sexual selection and the evolution of mating systems, In: Blum MS, Blum NA, editors. Sexual selection and reproductive competition in insects. New York: Academic Press. p. 19–80.

Boyer L, Lemichez E. 2004. Targeting of host-cell ubiquitin-like pathways by bacterial factors. Nat Rev Microbiol. 2:779–788.

Brawner WR III, Hill GE, Sundermann CA. 2000. Effects of coccidial and mycoplasmal infections on carotenoid-based plumage pigmentation in male house finches. Auk. 117:952–963.

Csermely P, Schnaider T, Soti C, Prohaszka Z, Nardai G. 1998. The 90-kDa molecular chaperone family: Structure, function, and clinical applications. A comprehensive review. Pharmacol Therapeutics. 79:129–168.

Dhondt AA, Badyaev AV, Dobson AP, Hawley DM, Driscoll MJL, Hochachka WM, Ley DH. 2006. Dynamics of mycoplasmal conjunctivitis in the native and introduced range of the host. EcoHealth. 3:95–102.

Farmer KL, Hill GE, Roberts SR. 2002. Susceptibility of wild songbirds to the house finch strain of *Mycoplasma gallisepticum*. J Wildl Dis. 41:317–325.

Folstad I, Karter AJ. 1992. Parasites, bright males, and the immunocompetence handicap. Am Nat. 139:603–622.

Ganapathy K, Bradbury JM. 2003. Effects of cyclosporin A on the immune responses and pathogenesis of a virulent strain of *Mycoplasma gallisepticum* in chickens. Avian Pathol. 32:495–502.

Hamilton WD, Zuk M. 1982. Heritable true fitness and bright birds: a role for parasites? Science. 218:384–386.

Hill GE. 1990. Female house finches prefer colourful males: sexual selection for a condition-dependent trait. Anim Behav. 40:563–572.

Hill GE. 1991. Plumage coloration is a sexually selected indicator of male quality. Nature. 350:337–339.

Hill GE. 1992. Proximate basis of variation in carotenoid pigmentation in male house finches. Auk. 109:1–12.

Hill GE. 2000. Energetic constraints on expression of carotenoid-based plumage coloration. Avian Biol. 31:559–566.

Hill GE. 2002. A red bird in a brown bag. New York: Oxford University Press.

Hill GE. 2006. Environmental regulation of ornamental coloration. In: Hill GE, McGraw KJ, editors. Bird coloration: mechanisms and measurements. Cambridge (MA): Harvard University Press. p. 507–560.

Hill GE. 2011. Condition dependent traits as signals of functionality of vital cellular processes. Ecol Lett. doi:10.1111/j.1461-0248.2011.01622.x

Hill GE. 2014. Cellular respiration: the nexus of stress, condition, and ornamentation. Integr Comp Biol. doi:10.1093/icb/icu029.

Hill GE, Farmer KL. 2005. Carotenoid-based plumage coloration predicts resistance to a novel parasite in the house finch. Naturwissenschaften. 92:30–34.

Hill GE, Fu X, Balenger SL, McGraw KJ, Girardeau M, Hood WR. 2013. Changes in concentrations of circulating heat shock proteins in wild songbirds in response to distinct environmental stressors. J Field Ornith. 84:416–424.

Hill GE, Johnson JD. 2012. The vitamin-A redox hypothesis: a biochemical basis for honest signaling via carotenoid pigmentation Am Nat. 180:E127–E150.

Hill GE, Farmer KL, Beck ML. 2004. The effect of mycoplasmosis on carotenoid plumage coloration in male house finches. J Exp Biol. 207:2095–2099.

Downloaded from http://beheco.oxfordjournals.org/ by guest on May 8, 2015

Hill GE, Inouye CY, Montgomerie R. 2002. Dietary carotenoids predict plumage coloration in wild house finches. Proc R Soc Lond Ser B. 269:1119–1124.

Johnson JD, Hill GE. 2013. Is carotenoid ornamentation linked to the inner mitochondria membrane potential? A hypothesis for the maintenance of signal honesty. Biochimie. 95:436–444.

Ley DH, Berkhoff JE, McLaren JM. 1996. *Mycoplasma gallisepticum* isolated from house finches (*Carpodacus mexicanus*) with conjunctivitus. Avian Dis. 40:480–483.

Luttrell MP, Fischer JR, Stallknecht DE, Kleven SH. 1996. Field investigation of *Mycoplasma gallisepticum* infections in house finches (*Carpodacus mexicanus*) from Maryland and Georgia. Avian Dis. 40:335–41.

McGraw KJ. 2006. Mechanics of carotenoid-based coloration. In: Hill GE, McGraw KJ, editors. Bird coloration: mechanisms and measurements. Cambridge (MA): Harvard University Press. p. 177–242.

Mohammed J, Frasca S Jr, Cecchini K, Rood D, Nyaoke AC, Geary SJ, Silbart LK. 2007. Chemokine and cytokine gene expression profiles in chickens inoculated with *Mycoplasma gallisepticum* strains R-low or GT5. Vaccine. 25:8611–8621.

Montgomerie R. 2006. Analyzing colors. In: Hill GE, McGraw KJ, editors. Bird coloration: mechanisms and measurements. Cambridge (MA): Harvard University Press. p. 90–147.

Mühlradt PF. 2002. Immunomodulation by mycoplasmas: artifacts, facts ad active molecules. In: Razin S, Herrmann R, editors. Molecular biology and pathogenicity of mycoplasmas. New York: Kluwer Academic/Plenum Publishers. p. 445–472.

Nolan PM, Hill GE, Stoehr AM. 1998. Sex, size, and plumage redness predict house finch survival in an epidemic. Proc R Soc Lond Ser B. 265:961–965.

Pockley AG, Muthana M, Calderwood S. 2008. The dual immunoregulatory roles of stress proteins. Trends Biochem Sci. 33:71–79.

Pointer MA, Prager M, Andersson S, Mundy NI. 2012. A novel method for screening a vertebrate transcriptome for genes involved in carotenoid binding and metabolism. Mol Ecol Res. 12:149–159.

Pryke SR, Lawes MJ, Andersson S. 2001. Agonistic carotenoid signaling in male red-collared widowbirds: aggression related to the colour signal of both the territory owner and model intruder. Anim Behav. 62:695–704.

Roberts SR, Nolan PM, Hill GE. 2001. Characterization of *Mycoplasma gallisepticum* infection in captive house finches (*Carpodacus mexicanus*) in 1998. Avian Dis. 45:70–75.

Rohner N, Jarosz DF, Kowalko JE, Yoshizawa M, Jeffery WR, Borowsky RL, Lindquist S, Tabin CJ. 2013. Cryptic variation in morphological evolution: HSP90 as a capacitor for loss of eyes in a cavefish. Science. 342:1372–1375.

Roulin A, Ducrest A-L. 2013. Genetics of colouration in birds. Sem Cell Dev Biol. 24:594–608.

Sorensen JG. 2010. Application of heat shock protein expression for detecting natural adaptation and exposure to stress in natural populations. Curr Zool. 56:703–713.

Thompson CW, Hillgarth N, Leu M, McClure HE. 1997. High parasite load in house finches (*Carpodacus mexicanus*) is correlated with reduced expression of a sexually selected trait. Am Nat. 149:270–294.

Udono H, Ichiyanagi T, Mizukami S, Imai T. 2009. Heat shock proteins in antigen trafficking – Implications on antigen presentation to T cells. Int J Hyperthermia. 25:617–625.

Walsh N, Dale J, McGraw KJ, Pointer MA, Mundy NI. 2012. Candidate genes for carotenoid coloration in vertebrates and their expression profiles in the carotenoid-containing plumage and bill of a wild bird. Proc R Soc Lond Ser B. 279:58–66.

Wang Z, Farmer KL, Hill GE, Edwards SV. 2006. A cDNA macroarray approach to parasite-induced gene expression in a songbird host: genetic response of house finches to experimental infection by *Mycoplasma gallisepticum*. Mol Ecol. 15:1263–73.

Young JC, Moarefi I, Hartl FU. 2001. Hsp90: a specialized but essential protein-folding tool. J Cell Biol. 154:267–273.

Downloaded from http://beheco.oxfordjournals.org/ by guest on May 8, 2015