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## Innate Immunity and the Evolution of Resistance to an Emerging Infectious Disease in a Wild Bird

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*(Article begins on next page)*

1 **Innate immunity and resistance to an emerging infectious disease in a wild bird**

2  
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9  
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11 microarray

12  
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19  
20 **Running title:** Evolution of resistance: role of innate immunity

21

22 **Abstract**

23 Innate immunity is expected to play a primary role in conferring resistance to novel infectious  
24 diseases. Despite this, few studies have attempted to examine its role in the evolution of  
25 resistance to emerging pathogens, instead concentrating on the role of acquired immunity  
26 (e.g. *Mhc* genes). Here we used experimental infections and cDNA microarrays to determine  
27 whether changes in the innate and/or acquired immune responses accompanied the  
28 emergence of resistance in eastern U.S. house finches (*Carpodacus mexicanus*) to a recent  
29 outbreak of conjunctivitis-causing bacterium (*Mycoplasma gallisepticum*- MG). Three days  
30 following experimental infection with MG, we observed differences in the transcriptional  
31 responses in spleens between House Finches from eastern or western US populations. In  
32 particular, birds from the western US, with no prior exposure to MG, down-regulated gene  
33 expression relative to controls, while those from the east, with a 12-year history of MG  
34 exposure, showed no expression change. This result is significant because, in poultry, MG is  
35 known to manipulate host immunity, suggesting that such manipulation also occurred in  
36 western birds only. Infected eastern birds then up-regulated genes associated with acquired  
37 immunity (cell-mediated immunity) 14 days after infection relative to controls, whereas birds  
38 from the western population retained similar expression patterns on day 14 as they did on day  
39 three. These observations indicate marked population differences in the temporal course of  
40 response to infection with MG, and suggest that innate immune processes were targets of  
41 selection in response to MG in the eastern U.S. population.

42

## 43 **Introduction**

44 Novel pathogens are powerful selective agents in humans (Diamond 1997) and other animals  
45 (Grenfell & Dobson 1995; Haldane 1949), and can have devastating effects on biodiversity  
46 (Benning *et al.* 2002; Lips *et al.* 2006). Studies simultaneously monitoring the emergence of  
47 an infectious disease in the wild and the associated changes in host populations are rare,  
48 leading to a reduced understanding of how hosts evolve immunity to novel pathogens,  
49 particularly in vertebrates. One exception involves the study of rapid evolution of disease  
50 resistance in European rabbit (*Oryctolagus cuniculus*) affected with myxomatosis in Australia  
51 (Kerr & Best 1998). The Myxoma virus was released in 1950 and spread rapidly throughout  
52 the susceptible Australian rabbit population. Within a few years however, resistance  
53 emerged, apparently mediated through escape from pathogen-induced immunosuppression  
54 which facilitated the development of an enhanced innate and then a specific cell-mediated  
55 immune response (Best & Kerr 2000). Although we know that wild vertebrate host  
56 populations can evolve resistance to novel pathogens rapidly (Bonneaud *et al.* 2011; Marshall  
57 & Fenner 1958), whether or not such resistance is mediated through initial changes to innate  
58 immunity as the study of rabbits would suggest is unclear.

59

60 Despite the potential for innate immunity to play a key role in the response to novel  
61 pathogens, the vast majority of studies in ecological immunology in vertebrates have focused  
62 on the acquired immune system (Acevedo-Whitehouse & Cunningham 2006; van der Most *et*  
63 *al.* 2011). The most likely reason for this trend is that most host-pathogen systems studied  
64 are assumed to be co-evolving. Unlike innate immunity, responses of acquired immunity are  
65 usually pathogen-specific and therefore represent a more targeted and effective defensive  
66 response, particularly against known pathogens (Janeway 2005). For example, of particular

67 interest in host-parasite co-evolution has been the role of the polymorphic *Mhc* genes in  
68 detecting foreign antigens and triggering pathogen-specific T-lymphocyte cytotoxicity and  
69 humoral immune responses (Piertney & Oliver 2006; Sommer 2005; Spurgin & Richardson  
70 2010). However, during the early stages of infection, pathogen-specific recognition alleles  
71 may either be absent or at such low frequencies in host populations that such populations are  
72 ill-equipped to deal with novel pathogens. Under such conditions, the spread of adaptive  
73 alleles may thus be slow and stochastic (Hedrick 2002; Wright 1955). By contrast, innate  
74 immunity comprises immediate, non-specific immune processes that are triggered when  
75 pattern recognition receptors detect a limited repertoire of conserved but common microbial  
76 patterns (e.g., LPS) (Janeway 1989). As a result, innate immunity provides the first line of  
77 protection against most pathogenic attacks and can stem infections while pathogen-specific  
78 processes are being activated (Janeway 2005). As such, we might expect innate immunity to  
79 play a particularly important role during outbreaks of novel infectious diseases. This is  
80 particularly true of pathogens that are able to manipulate and avoid immune detection, since  
81 detection by the acquired immune system (e.g., by *Mhc* molecules) requires their prior  
82 recognition and presentation by cells of the innate immune system (e.g., macrophages,  
83 dendritic cells) (Iwasaki & Medzhitov 2010). Thus, given the primary role of innate  
84 immunity in non-specifically fighting infections and in regulating acquired immune  
85 responses, it is likely that the innate immune processes are paramount in driving resistance to  
86 novel pathogens, particularly those that avoid immune detection.

87

88 Here we make use of the natural epizootic of conjunctivitis caused by the bacterium  
89 *Mycoplasma gallisepticum* (MG) in a North American songbird, the House finch  
90 (*Carpodacus mexicanus*) (Dhondt *et al.* 1998; Fischer *et al.* 1997), to investigate the

91 contribution of innate and acquired immunity to the evolution of resistance to a novel  
92 pathogen. Mycoplasmosis was first reported in house finches in Maryland in 1994 (Ley  
93 1996). Following outbreak, the disease spread rapidly across eastern populations of house  
94 finches in North America. The severity of MG as a house finch pathogen early in the  
95 epizootic was confirmed by high mortality rates of naturally- and experimentally-infected  
96 finches maintained in captivity (Farmer *et al.* 2002; Luttrell *et al.* 1998; Roberts *et al.* 2001a).  
97 In the wild, hundreds of millions of birds were estimated to have died between 1994 and  
98 1998 (Nolan *et al.* 1998), causing a significant decline in the abundance of house finches over  
99 the entire eastern portion of their range (Hochachka & Dhondt 2000). The prevalence of MG  
100 in house finches subsequently declined (Hartup *et al.* 2001; Roberts *et al.* 2001b) and  
101 evidence now suggests that MG has reached endemic levels in eastern North America, at  
102 least in part due to the spread of host resistance within 12 years of exposure to MG  
103 (Bonneaud *et al.* 2011).

104

105 *Mycoplasma* bacteria are known for effectively evading and manipulating host immune  
106 defenses (for a review see (Razin *et al.* 1998). For example, MG maintains a high diversity of  
107 cell surface molecules (Chambaud *et al.* 1999), including surface lipoproteins, and can vary  
108 its antigenic composition at the cell surface in response to environmental cues (Baseggio *et*  
109 *al.* 1996; Markham *et al.* 1998). Such antigenic variation allows mycoplasmas to be resistant  
110 to phagocytosis in susceptible hosts (Marshall *et al.* 1995). Immuno-modulatory effects  
111 include the ability to induce an inflammatory response at the site of infection (Ganapathy &  
112 Bradbury 2003; Gaunson *et al.* 2006), causing host lesions (Ley 2008), as well as the ability  
113 to suppress other components of host immunity (Javed *et al.* 2007). For example,  
114 simultaneous inoculation of poultry with MG and *Haemophilus gallinarum* (Matsuo *et al.*

115 1978) or avian pneumovirus (Naylor *et al.* 1992) has been found to lower the humoral  
116 antibody response to both *H. gallinarum* and pneumovirus in chickens and turkeys,  
117 respectively. Finally, MG infection is associated with suppressed T cell activity two weeks  
118 after infection (Ganapathy & Bradbury 2003; Gaunson *et al.* 2000).

119

120 To examine the contributions of innate and acquired immunity to the evolution of resistance  
121 to MG in house finches, we conducted an infection experiment and examined transcriptional  
122 responses elicited in the spleen, an important tissue for the organization of both innate and  
123 acquired immunity (Mebius & Kraal 2005). Infection with pathogens is known to induce  
124 transcriptional responses in hosts (Jenner & Young 2005) and such responses can differ  
125 between individuals displaying varying levels of resistance to infection (Marquis *et al.* 2008).  
126 Investigating differences in gene expression profiles between resistant and susceptible hosts  
127 in response to experimental infection might therefore offer new insights into the genetic basis  
128 underlying immunity (Sarson *et al.* 2008; van der Sar *et al.* 2009). In our study, finches  
129 originated from either eastern U.S. (Alabama) populations, which have coexisted with MG  
130 since the mid-1990s and show evidence of having evolved resistance, or western U.S.  
131 (Arizona) populations with no prior exposure to MG (Bonneaud *et al.* 2011). Gene  
132 expression changes between infected and control finches were measured three and 14 days  
133 after experimental infection. Although immune processes three and 14 days post-infection  
134 will generally reflect innate and acquired activity, respectively (Farmer *et al.* 2002; Gaunson  
135 *et al.* 2000; Hickman-Davis *et al.* 1998; Lai *et al.* 1987), the genes that underpin these  
136 processes may both be expressed sharply after, and continue throughout, infection (Caipang  
137 *et al.* 2009; Raida & Buchmann 2008; Sarson *et al.* 2008; van der Sar *et al.* 2009). Thus,  
138 investigating the role of innate and acquired immunity in the evolution of resistance to MG

139 using patterns of gene expression profiles in transcriptional responses to MG-infection will  
140 require testing predictions regarding temporal versus geographical differences.

141

142 We make two broad predictions regarding the role of innate and acquired immune responses  
143 in the evolution of resistance to MG in eastern house finches. First, our results would suggest  
144 that MG has selected on innate immunity if: (1) eastern and western populations differed in  
145 the transcriptional changes observed between control and MG-infected finches three days  
146 post-infection; (2) gene expression differences involved significant gene down-regulation in  
147 western but not eastern finches; and (3) genes associated with acquired immunity were up-  
148 regulated on day 14 only. These predictions arise because transcriptional differences  
149 between populations in the early stages of experimental infection would suggest that early-  
150 acting innate immune processes differ between populations, and down-regulation is expected  
151 in Arizona due to the immuno-modulatory effects of MG infection. However, this scenario  
152 would unambiguously support the hypothesis of selection on innate immunity only if genes  
153 known to be associated with acquired immunity were not differentially expressed at an early  
154 stage of infection. Second, by contrast, our results would suggest a sole role of acquired  
155 immunity in the resistance of eastern bird to MG if transcriptional changes only differed  
156 between populations fourteen days after infection and involved the up-regulation of genes  
157 associated with acquired immunity in eastern finches.

158

## 159 **Material and Methods**

### 160 *Experimental infection*

161 In January and February 2007, we captured male house finches from two geographically  
162 distant locations: southeastern Arizona in the western U.S. which was outside the 2007-range

163 of MG; and southern Alabama in the eastern U.S., where finches had co-existed with MG for  
164 12 years. Sampling was conducted at 3 different suburban sites in both states: in Arizona,  
165 sites were 1-2 km apart and the birds were captured over 3 days; in Alabama, sites were 10-  
166 103 km apart and the birds were captured over 30 days. Following capture, birds were  
167 immediately transported by plane from Arizona ( $N=37$ ) and by car within Alabama ( $N=64$ ),  
168 and established in aviaries at Auburn University, Alabama. Finches were held in cages 0.5 m  
169 x 0.5 m with two birds per cage for the duration of the study. Cages were kept indoors, in  
170 temperature-controlled rooms with natural light through windows (day-length was  
171 unregulated but comparable to the locales from which the birds were captured). Captive  
172 finches were fed sunflower seed, brown and white millet, grit, and water *ad libitum*, as well  
173 as apple slices and crushed eggshells weekly. The housing conditions, food, and day-length  
174 regime were identical for birds from both populations, and represented novel conditions for  
175 birds from both populations.

176

177 To confirm that the finches had not been infected with MG prior to our study, individuals  
178 from Alabama and Arizona were quarantined in separate rooms for the first month.  
179 Following quarantine, birds were weighed ( $\pm 0.1$ g) and had a blood sample taken via brachial  
180 venipuncture ( $\sim 60$   $\mu$ l of whole blood). Whole blood was tested for MG antibodies using  
181 serum plate agglutination assay (SPA), a reliable means of determining prior exposure to  
182 MG, (Luttrell *et al.* 1996). All birds in the study were further tested for exposure to MG via  
183 amplification of MG DNA from choanal and conjunctival swabs (Roberts *et al.* 2001a).  
184 Twelve birds from the Alabama population were removed from the experiment when they  
185 showed evidence of exposure to MG (8 were symptomatic at capture, 1 developed symptoms  
186 during quarantine, and 3 were seropositive for MG-antibodies). In addition, a further 9 from

187 Arizona and 20 from Alabama were used in a different experiment, leaving 28 Arizona birds  
188 and 32 Alabama birds in this study.

189

190 Birds were either kept as controls or infected via ocular inoculation with 20  $\mu$ l of culture  
191 containing  $1 \times 10^4$  to  $1 \times 10^6$  color changing units/ml of an early 2007 Auburn MG isolate.  
192 Control birds were sham infected using sterile SP4 medium (Whitcomb 1983). Control  
193 ( $N=11$  birds from Arizona and 9 from Alabama) and infected birds were maintained under  
194 identical conditions, but in separate rooms of an aviary. Birds were euthanized three days  
195 ( $N=6$  from Arizona and  $N=11$  from Alabama) and 14 days ( $N=11$  from Arizona and  $N=12$   
196 from Alabama) after treatment. The spleens and the conjunctiva from all birds were removed  
197 immediately after euthanization, stored in RNAlater (Ambion), and placed at  $-80^\circ\text{C}$ .

198

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

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

206

207 The samples were hybridized onto a microarray printed with a selection of cDNA clones  
208 from two subtraction suppression hybridization libraries (Bonneaud *et al.* 2011). These  
209 libraries are enriched in clones differentially expressed between MG-infected and control  
210 house finches 2-weeks post-infection ( $N=16,512$  clones) (Wang et al. 2006). Using libraries

211 enriched in cDNA differentially expressed 14 days post-infection increases the probability  
212 that both innate and acquired immune processes have been activated (Janeway 2005). Of all  
213 the clones present in the libraries, 220 were previously identified as significantly  
214 differentially expressed between infected and controls using a macroarray approach (Wang *et al.*  
215 *al.* 2006). The microarray consisted of unique amplicons of these 220 clones, as well as  
216 amplicons of 694 randomly selected clones from the enriched libraries (Bonneaud *et al.*  
217 2011). Additionally, it contained five house finch housekeeping genes (*Actin related protein*  
218 *2/3*, *ATP synthetase*, *ATPase VI subunit G1*, *Basic transcription factor 3*, *Calmodulin 2*) and  
219 11 *E. coli* housekeeping genes (*arcA*, *aroE*, *dnaE*, *gapA*, *gnd*, *icdA*, *pgm*, *polB*, *putin*, *trpA*,  
220 *trpB*; (Hommais *et al.* 2005; Noller *et al.* 2003)) to facilitate normalization procedures. All  
221 clones were printed twice on each grid and each grid was replicated twice on each half  
222 microarray slide. We used a common reference design (Yang & Speed 2002), in which we  
223 pooled 2 to 5 spleens from birds from the same population in the same treatment to generate  
224 enough mRNA for microarray hybridizations and hybridized two pools for each treatment  
225 from each population. Pools were labeled using Cy5 dye and hybridized against a common  
226 reference, made by pooling an aliquot of all the individual samples from all treatments and  
227 labeled with Cy3.

228

229 We used the software package GenePix to yield log base-2 ( $\log_2$ ) measurements for mean  
230 fluorescence intensities for each dye channel in each spot on the array and to flag low quality  
231 spots. We normalized the log base-2 measurements of mean fluorescence intensities for each  
232 dye channel in each spot on the array using R software (<http://www.r-project.org>), and a  
233 Matlab interface (MArray), which allows results to be graphically presented and normalized  
234 (Wang *et al.* 2002). Normalized signal ratios were then fitted to Linear Model for Microarray

235 data (LIMMA) in an R Bioconductor package; LIMMA is similar to a General Linear Model  
236 but provides False Discovery Rate (FDR)-adjusted probability values of differential  
237 expression. This approach controls for multiple comparisons in microarray data,  
238 substantially reducing the probability of discovering false positives (Type I errors)  
239 (Benjamini & Hochberg 1995). The ratios generated by the external spike-ins were used for  
240 quality control. To control for within-hybridization spatial variation, we compared the signal  
241 from the 2 replicated grids. To control for between-slide differences, we compared the  
242 signals from the *E. coli* external spike-ins, the house finch housekeeping genes and the  
243 common reference on the different slides. All clones were considered to be differentially  
244 expressed only when both replicates on the array displayed a significant deviation from the  
245 mean of the standard. All differentially expressed clones were sequenced on an ABI 377  
246 sequencer. Forward and reverse sequences generating a BLAST hit with an e-value  $< 1 \times 10^{-20}$   
247 and with more than 100 nucleotides were categorized by their vertebrate homologues, while  
248 all other genes were considered to be unknown. Gene ontology category and function were  
249 determined using Harvester (<http://harvester.fzk.de/harvester/>).

250

### 251 *Comparisons*

252 To test our predictions, we made four comparisons of transcriptional responses to MG-  
253 infection between finches (Figure 1A). We compared expression differences between  
254 infected birds on day three post-infection vs. controls in Arizona (1) and Alabama (2), as well  
255 as those differences between control and experimental birds on day 3 with those on day 14 in  
256 Arizona (3) and Alabama (4). Differences in gene expression patterns were analyzed using  
257 comparisons of observed versus expected frequencies in binomial test and contingency tables  
258 (comparing two or more than two independent frequencies, respectively) or McNemar's and

259 Cochran's Q test (when comparing two, or more than two, non-independent frequencies,  
260 respectively; e.g. when frequencies are based on the same sample of subjects or matched-pair  
261 samples such as before and after treatment).

262

## 263 **Results**

264 We found 105 clones that were significantly differentially expressed in this study, of which  
265 73 were differentially expressed three days after infection and 99 were differentially  
266 expressed 14 days after infection. Sequencing these clones revealed 25 vertebrate orthologs  
267 (Figure 1B): 13 and 24 which were differentially expressed three and 14 days after infection,  
268 respectively. All other clones were unknown. Gene ontology categories and primary  
269 functions of the 25 genes included immunity (6 genes), redox metabolism (3), metabolism  
270 (1), signal transduction (4), stress (1), cytoskeleton (4), transcription/translation (3), transport  
271 (2), and cell differentiation (1). Given that all of these genes are differentially expressed as a  
272 result of experimental infection, it is likely they all play some role in the response to  
273 infection. Indeed, in addition to the 6 genes with direct immune function (*T-cell*  
274 *immunoglobulin and mucin domain containing 4*, *MHC class II-associated invariant chain*  
275 *Ii*, *programmed death ligand 1*, *lectin galactoside-binding soluble 2 protein*, *neutrophil*  
276 *cytosolic factor 4*, *complement factor H*), three of the 'non-immune' genes above have been  
277 shown to have auxiliary immune function (*thioredoxin* (Nordberg & Arner 2001), *RhoA*  
278 *GTPase* (Scheele *et al.* 2007), *lymphocyte cytosolic protein* (Samstag *et al.* 2003)) (Figure  
279 1B). We can rule out the possibility that our results are due to differences in cDNA quality or  
280 abundance between samples due to our extensive use of within-and between-slide controls  
281 (see Methods).

282

283 All predictions that selection has acted on innate immunity only, or on both innate and  
284 acquired immunity, were upheld. Three days post-infection, 13 of the 25 genes identified  
285 displayed significant differences in expression between infected and control birds from  
286 Arizona (comparison 1), but none did between such birds from Alabama (comparison 2)  
287 (Figure 1B, C) (two-sample binomial test=3.85,  $P<0.001$ ). In addition, 85% of those 13  
288 genes differentially expressed on day three between infected and control birds in Arizona  
289 were down-regulated (one-sample binomial test,  $P = 0.02$ ; Figure 1B, C). Finally, in Arizona  
290 birds, gene expression profiles between experimental and control birds remained similar on  
291 days three and 14 post-treatment (13 genes differentially expressed in comparison 1 and 20 in  
292 comparison 3: two-sample binomial test=-1.48,  $P=0.14$ ), and there was no change in the  
293 proportion of genes that were down-regulated between the two time points (McNemar's test,  
294  $\chi^2=0.14$ ,  $P = 0.71$ ). By contrast, in Alabama, a significantly greater number of genes were  
295 expressed in infected birds on day 14 than on day three (0 genes in comparison 2, 11 in  
296 comparison 4: two-sample binomial test=-3.51,  $P<0.001$ ), and eight of these 14 genes were  
297 up-regulated. Importantly, of these eight genes differentially expressed on day 14 in  
298 Alabama, one was identified as having a role in innate immunity (*neutrophil cytosolic factor*  
299 *4*) and two in acquired immunity (*T-cell immunoglobulin* and *MHC class II associated*  
300 *invariant chain*), and none was differentially expressed on day 3. This latter result means  
301 that population differences in expression patterns 3 days post-infection are unlikely to be  
302 attributed to acquired immune processes.

303

## 304 **Discussion**

305 We have shown recently that eastern U.S. populations of house finches evolved resistance to  
306 a devastating outbreak of MG over a 12-year period (Bonneaud *et al.* 2011). Here we use

307 microarray analysis and experimental infections in finches from MG-exposed eastern U.S.  
308 (Alabama) and unexposed western U.S. (Arizona) populations to investigate whether changes  
309 to innate and/or acquired immunity have accompanied this evolutionary event. Relative to  
310 controls, gene expression profiles of birds from Arizona versus Alabama differed both three  
311 and 14 days following experimental infection, with infected birds from Arizona showing  
312 significant down-regulation of gene expression patterns on both days compared to those from  
313 Alabama. Moreover, while gene expression profiles were similar on days three and 14 in  
314 Arizona finches, in Alabama finches, profiles differed significantly between day three and 14.  
315 This change in gene expression patterns in Alabama finches was generated by the up-  
316 regulation of acquired immune processes by day 14 but not on day three. Inter-population  
317 differences between infected and control birds on days three and 14 were therefore likely due  
318 to differences in innate and acquired immune activity. From these observations, we suggest  
319 that mutations affecting innate immunity only, or both innate and acquired immunity, have  
320 accompanied the evolution of resistance to MG.

321

322 The conclusion that mutations affecting innate immunity played a role in the evolution of  
323 resistance to MG is based on our upholding of three predictions (see Introduction). These  
324 were: (1) relative to controls, infected birds from Arizona and Alabama displayed distinct  
325 transcriptional responses in the early stages of experimental infection; (2) expression patterns  
326 in Alabama were consistent with increased resistance to MG; and (3) genes associated with  
327 acquired immunity were only up-regulated after population differences in transcription were  
328 first observed. These observations also allowed us to reject the hypothesis that mutations  
329 associated with acquired immunity alone led to the evolution of resistance to MG among  
330 eastern U.S. house finches. Nevertheless, mutations associated with acquired immune

331 processes, in addition to those associated with innate immune processes, may have played a  
332 role in the evolution of resistance, as evidenced by the transcriptional differences of infected  
333 versus control birds between the two populations on day 14, and within Alabama between  
334 days three and 14.

335

336 Evidence from laboratory mice and rats also suggests a role of both innate and acquired  
337 immunity in fighting infections with *Mycoplasmas*, but with innate immunity playing a  
338 predominant role in fighting initial infections (Hickman-Davis 2002). For example, while  
339 acquired immunity appears to be implicated in controlling the spread of *M. pulmonis* within  
340 the body, innate immunity is important for resistance against acute infections (Cartner *et al.*  
341 1998). Natural killer cells and macrophages, which are important actors of innate immunity,  
342 have been shown to play important roles in conferring resistance to *M. pulmonis* (Hickman-  
343 Davis *et al.* 1997; Lai *et al.* 1990). In addition, phagocytosis, bacterial killing and the release  
344 of reactive nitrogen species by macrophages during *M. pulmonis* and *M. pneumonia*  
345 infections seem to be facilitated by collectins, such as surfactant-associated proteins A  
346 (Hickman-Davis *et al.* 1998; Kalina *et al.* 2000; Marshall *et al.* 1995), which represent a  
347 major group of pattern recognition proteins of the innate immune system (van de Wetering *et*  
348 *al.* 2004). Surfactant-associated proteins A are encoded by polymorphic genes (reviewed in  
349 (Floros *et al.* 2009; Ledford *et al.*), and both limit inflammatory responses and interact with T  
350 cells, making them particularly interesting candidate genes to examine in the context of the  
351 evolution of resistance to MG in eastern U.S. house finches.

352

353 Although studies of the response of mammalian hosts to *Mycoplasmas* suggest a role of both  
354 innate and acquired immunity in conferring resistance, the evolutionary origins of resistance

355 to MG could be associated with changes in gene(s) implicated in innate immunity only, given  
356 that innate immune processes both precede and play a critical role in the activation of  
357 acquired processes (Iwasaki & Medzhitov 2010). Under this hypothesis, any population  
358 differences in acquired immunity may simply be a consequence of differences in innate  
359 immune activity. Hence, although we are not in a position to distinguish whether mutations  
360 associated with innate, or with both innate and acquired, immunity have led to the evolution  
361 of resistance to MG in eastern house finches, the transcriptional differences we observed on  
362 day 14 may result from a single mutation affecting innate immunity and allowing eastern  
363 finches to subsequently trigger an acquired immune response. Our results are reminiscent of  
364 those obtained from similar experimental infections of wild rabbits with the *myxoma* virus  
365 (Best & Kerr 2000). Resistant rabbits had elevated immune responses within four days post-  
366 infection, in advance of the subsequent increased cell-mediated immune response at least six  
367 days after infection. The increased resistance of populations of rabbits having experienced  
368 the *myxomatosis* outbreak was therefore hypothesized to be mediated by enhanced innate  
369 immune activity, which subsequently allowed the development of a specific cell-mediated  
370 immune response (Best & Kerr 2000). While mutations arising in genes associated with both  
371 innate and acquired immunity may have been subject to natural selection, a more  
372 parsimonious scenario may be that a change in the frequency of a single mutation affecting  
373 innate immune processes has been primarily responsible for the evolution of resistance to  
374 MG. The speed with which resistance evolved in eastern house finches (Bonneaud *et al.*  
375 2011) and the rarity of mutations conferring phenotypic advantages in evolving populations  
376 (Blount *et al.* 2008), suggests that selection is unlikely to have simultaneously favored the  
377 spread of two or more distinct pre-existing alleles, but further work is required to test this  
378 hypothesis.

379

380 Although all of the transcriptional changes that we observed occurred in response to the  
381 experimental infection, and hence might play a role in resistance, we identified six genes that  
382 are known to have a direct role in immunity in model organisms and humans, and three genes  
383 known to play an auxiliary role in immunity (see Table 1 for full details of gene functions  
384 and associated references). Of the six immune genes, three encode proteins that are directly  
385 involved in innate immunity and implicated in phagocytosis-induced superoxide production  
386 and/or control of inflammation or complement-mediated immunity: *neutrophil cytosolic*  
387 *factor 4* was up-regulated on day 14 in Alabama finches; *lectin galactoside-binding soluble 2*  
388 *protein (galectin)* was down-regulated on day 14 in Arizona finches; and *complement factor*  
389 *H* was up-regulated on day 14 in Arizona finches; surprisingly, none was differentially  
390 expressed on day three. In addition to the *galectin* gene above which also has direct  
391 involvement in acquired immunity, *T-cell immunoglobulin and mucin domain containing 4*,  
392 which plays a role in T-cell activation, was up-regulated in Alabama finches on day 14,  
393 *programmed death ligand 1*, which regulates T-cell activation and tolerance, was down-  
394 regulated in Arizona finches on day 14, and *MHC class II-associated invariant chain Ii*,  
395 which plays a role in the assembly of MHC class II molecules, was up-regulated in Alabama  
396 finches on day 14 and down-regulated in Arizona on both days. Finally, *thioredoxin* and  
397 *RhoA GTPase* which both have auxiliary function in innate immunity (antioxidant activities,  
398 regulation and coordination of the innate immune response, respectively) were down  
399 regulated on days three and 14 in Arizona finches and up-regulated in Alabama finches on  
400 day 14, while *lymphocyte cytosolic protein*, which has auxiliary function in acquired  
401 immunity (stabilization of actin filaments during T-cell migration) was down-regulated in  
402 Arizona finches on both days and up-regulated in Alabama finches on day 14.

403

404 MG infection is known to cause the suppression of certain immune components in the initial  
405 stages of infection in chickens as evidenced by the significant down-regulation of cytokines  
406 (CCL20, IL8 and IL12) as early as 24 hours after exposure (Mohammed *et al.* 2007). These  
407 effects can last up to 8 days following infection (Mohammed *et al.* 2007). The expression  
408 profiles above suggest that Arizona finches were immune-suppressed throughout the  
409 experimental infection, with the majority of genes being down-regulated, including 3 genes  
410 associated with immunity on day three and five on day 14. Interestingly, the only immune  
411 gene that was up-regulated in infected finches from Arizona (*complement factor H*) has been  
412 found to restrict the activation of the complement cascade in humans (de Cordoba & de Jorge  
413 2008) and hence exhibits a direction of expression change consistent with the suppression of  
414 immune activity. Conversely, Alabama finches displayed evidence of resistance to immune  
415 manipulation as no immune related genes were down-regulated on day three or day 14.  
416 Finally, in line with the study of the rabbits/myxomatosis system wherein immunity against  
417 myxomatosis was associated with increased cell-mediated (i.e. T helper-cell activity) rather  
418 than humoral (i.e. antibody) responses (Best and Kerr 2000), we found that the two immune  
419 genes up-regulated on day 14 in Alabama were associated with cell-mediated immunity and  
420 that no differentially expressed genes identified were associated with humoral responses.  
421 Thus, our results suggest that resistance to MG evolved in the eastern U.S. via the ability to  
422 mount an innate immune response followed by a cell-mediated immune response against  
423 MG.

424

425 Resistance can evolve via increased host ability to physiologically limit pathogen invasion  
426 upon contact (avoidance), to clear infections (recovery), or to suffer the costs associated with

427 the presence of the pathogen (tolerance) (Boots & Bowers 1999). Whether clearance of  
428 infection is mediated by innate or acquired immune processes should depend on  
429 characteristics of both the host and the pathogen, such as host lifespan, pathogen transmission  
430 rate and pathogenicity, and host recovery rate (Boots & Bowers 2004). In the initial stages  
431 of a novel and severe epizootic outbreak, however, innate immune mechanisms conferring  
432 increased resistance may be the target of selection, even if natural selection ultimately leads  
433 to the evolution of highly-specific acquired immune processes. Our results highlight the  
434 importance of identifying not only the genetic correlates of adaptation, but also the molecular  
435 and cellular processes underlying phenotypic change to better understand how wild  
436 populations respond to natural selection (Manceau *et al.* 2011; Shapiro *et al.* 2004). In  
437 addition, we showed that the same immune processes appear to be adopted by different  
438 species in response to related pathogens, suggesting that the pathways favored by natural  
439 selection may be analogous across taxa. Finally, although previous studies of temporal  
440 transcriptional changes have been used to identify the immune processes associated with  
441 increased resistance to infectious diseases in both domestic and laboratory animals (Raida  
442 and Buchmann 2008; Sarson *et al.* 2008), ours is the first to do so in a wild population known  
443 to have evolved disease resistance under pathogen-driven natural selection.

444

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457

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656

657

658 **Figure legends**

659 **Figure 1:** Comparisons and patterns of splenic gene expression. (A) Schematic of the  
660 analytical comparisons made: (1) infected on day three post-inoculation *vs.* controls in MG-  
661 unexposed Arizona; (2) infected on three post-inoculation *vs.* controls in MG-exposed  
662 Alabama; (3) infected on day fourteen post-inoculation *vs.* controls in Arizona; (4) infected  
663 day fourteen post-inoculation *vs.* controls in Alabama. Comparisons (3) and (4) were  
664 previously published in Bonneaud et al (2011). (B) Heat map of gene expression patterns for  
665 the 25 genes in comparisons 1-4 above (1<sup>st</sup> treatment/population *vs.* 2<sup>nd</sup> one). The 25 genes  
666 are all those showing differential expression in at least one comparison (1-4) and of known  
667 function. Values in red and green indicate significantly higher and lower expression levels,  
668 respectively, in comparisons 1-4 above, with bright colors reflecting at least a 3-fold  
669 difference in magnitude and values in black indicating no difference. Gene functions and  
670 identities are shown on the right; asterisks indicate genes with an identified auxiliary immune  
671 function. (C) Total number of genes of known function up-regulated (black) and down-  
672 regulated (white) in infected *vs.* control finches in the comparisons 1-4 above.

673 **Fig. 1**

