DIGITAL ACCESS TO SCHOLARSHIP AT HARVARD

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

Citation	Bonneaud, Camille, Susan L. Balenger, Jiangwen Zhang, Scott V. Edwards, and Geoffrey E. Hill. 2012. "Innate immunity and the evolution of resistance to an emerging infectious disease in a wild bird." Molecular Ecology 21 (11) (June 19): 2628-2639. doi:10.1111/j.1365-294X.2012.05551.x. http://dx.doi.org/10.1111/j.1365-294X.2012.05551.x.
Published Version	doi:10.1111/j.1365-294X.2012.05551.x
Accessed	April 17, 2018 4:27:43 PM EDT
Citable Link	http://nrs.harvard.edu/urn-3:HUL.InstRepos:11688779
Terms of Use	This article was downloaded from Harvard University's DASH repository, and is made available under the terms and conditions applicable to Open Access Policy Articles, as set forth at http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#OAP

(Article begins on next page)

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

2

3   Camille Bonneaud[1,2*], Susan L. Balenger[3], Scott V. Edwards[1], Geoffrey E. Hill[3]

4

5   1. Department of Organismic and Evolutionary Biology, Harvard University,

6   Cambridge, U.S.A.

7   2. Station d'Ecologie Expérimentale du CNRS (USR2936), 09200 Moulis, France

8   3. Department of Biological Sciences, Auburn University, Auburn, U.S.A.

9

12

13   * corresponding author: Camille Bonneaud

14   Station d'Ecologie Expérimentale du CNRS (USR2936)

15   09200 Moulis, France

16   Email: bonneaud@dr14.cnrs.fr

17   Tel. +33 5 61 04 03 62

18   Fax. +33 5 61 96 08 51

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

Introduction

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

Despite the potential for innate immunity to play a key role in the response to novel pathogens, the vast majority of studies in ecological immunology in vertebrates have focused on the acquired immune system (Acevedo-Whitehouse & Cunningham 2006; van der Most *et al.* 2011). The most likely reason for this trend is that most host-pathogen systems studied are assumed to be co-evolving. Unlike innate immunity, responses of acquired immunity are usually pathogen-specific and therefore represent a more targeted and effective defensive 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 (*Caropdacus 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 ($\pm$ 0.1g) and had a blood sample taken via brachial

180   venipuncture (~60 μl 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 μl of culture

191 containing 1 x 10^4 to 1 x 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°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 -80ºC until further processing.

206

207 The samples were hybridized onto a microarray printed with a selection of cDNA clones

208 from two substraction 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*

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 V1 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

15

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 pathogenecity, 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

457

458 **Literature cited**

459 Acevedo-Whitehouse K, Cunningham AA (2006) Is MHC enough for understanding wildlife

460   immunogenetics? *Trends in Ecology & Evolution,* **21**, 433-438.

461 Baseggio N, Glew MD, Markham PF, Whithear KG, Browning GF (1996) Size and genomic

462   location of the pMGA multigene family of Mycoplasma gallisepticum. *Microbiology-*

463   *Uk,* **142**, 1429-1435.

464 Benjamini Y, Hochberg Y (1995) Controlling the False Discovery Rate - a Practical and

465   Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society Series*

466   *B-Methodological,* **57**, 289-300.

467 Benning TL, LaPointe D, Atkinson CT, Vitousek PM (2002) Interactions of climate change

468   with biological invasions and land use in the Hawaiian Islands: Modeling the fate of

469   endemic birds using a geographic information system. *Proceedings of the National*

470   *Academy of Sciences of the United States of America,* **99**, 14246-14249.

471 Best SM, Kerr PJ (2000) Coevolution of host and virus: The pathogenesis of virulent and

472   attenuated strains of myxoma virus in resistant and susceptible European rabbits.

473   *Virology,* **267**, 36-48.

474   Blount ZD, Borland CZ, Lenski RE (2008) Historical contingency and the evolution of a key

475   innovation in an experimental population of *Escherichia coli*. *Proceedings of the*

476   *National Academy of Sciences of the United States of America,* **105**, 7899-7906.

477   Bonneaud C, Balenger S, Russell AF*, et al.* (2011) Rapid evolution of disease resistance is

478   accompanied by functional changes in gene expression in a wild bird. *Proceedings of*

479   *the National Academy of Sciences of the United States of America,* **108**, 7866-7871.

480   Boots M, Bowers RG (1999) Three mechanisms of host resistance to microparasites -

481   Avoidance, recovery and tolerance - Show different evolutionary dynamics. *Journal*

482   *of Theoretical Biology,* **201**, 13-23.

483   Boots M, Bowers RG (2004) The evolution of resistance through costly acquired immunity.

484   *Proceedings of the Royal Society of London Series B-Biological Sciences,* **271**, 715-

485   723.

486   Caipang CMA, Brinchmann MF, Kiron V (2009) Profiling gene expression in the spleen of

487   Atlantic cod, *Gadus morhua* upon vaccination with *Vibrio anguillarum* antigen.

488   *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology,*

489   **153**, 261-267.

490   Cartner SC, Lindsey JR, Gibbs-Erwin J, Cassell GH, Simecka JW (1998) Roles of innate and

491   adaptive immunity in respiratory mycoplasmosis. *Infection and Immunity,* **66**, 3485-

492   3491.

493   Chambaud I, Wroblewski H, Blanchard A (1999) Interactions between mycoplasma

494   lipoproteins and the host immune system. *Trends in Microbiology,* **7**, 493-499.

495   de Cordoba SR, de Jorge EG (2008) Translational mini-review series on complement factor

496   H: Genetics and disease associations of human complement factor H. *Clinical and*

497   *Experimental Immunology,* **151**, 1-13.

498 Dhondt AA, Tessaglia DL, Slothower RL (1998) Epidemic mycoplasmal conjunctivitis in
499   house finches from Eastern North America. *Journal of Wildlife Diseases, 34*, 265-
500   280.

501 Diamond J (1997) *Guns, germs, and steel* W. W. Norton & Company, New York, U.S.A.

502 Farmer KL, Hill GE, Roberts SR (2002) Susceptibility of a naive population of house finches
503   to *Mycoplasma gallisepticum*. *Journal of Wildlife Diseases, 38*, 282-286.

504 Fischer JR, Stallknecht DE, Luttrell MP, Dhondt AA, Converse KA (1997) Mycoplasmal
505   conjunctivitis in wild songbirds: The spread of a new contagious disease in a mobile
506   host population. *Emerging Infectious Diseases, 3*, 69-72.

507 Floros J, Wang GR, Mikerov AN (2009) Genetic complexity of the human innate host
508   defense molecules, surfactant protein A1 (SP-A1) and SP-A2-impact on function.
509   *Critical Reviews in Eukaryotic Gene Expression, 19*, 125-137.

510 Ganapathy K, Bradbury JM (2003) Effects of cyclosporin A on the immune responses and
511   pathogenesis of a virulent strain of *Mycoplasma gallisepticum* in chickens. *Avian
512   Pathology, 32*, 495-502.

513 Gaunson JE, Philip CJ, Whithear KG, Browning GF (2000) Lymphocytic infiltration in the
514   chicken trachea in response to *Mycoplasma gallisepticum* infection. *Microbiology-Uk,
515   146*, 1223-1229.

516 Gaunson JE, Philip CJ, Whithear KG, Browning GF (2006) The cellular immune response in
517   the tracheal mucosa to *Mycoplasma gallisepticum* in vaccinated and unvaccinated
518   chickens in the acute and chronic stages of disease. *Vaccine, 24*, 2627-2633.

519 Grenfell BT, Dobson AP (1995) *Ecology of infectious diseases in natural populations*
520   Cambridge University Press, Cambridge.

521 Haldane JBS (1949) Disease and evolution. *La Ricerca Scientifica Supplemento A, 19*, 68-76.

522   Hartup BK, Bickal JM, Dhondt AA, Ley DH, Kollias GV (2001) Dynamics of conjunctivitis

523   and *Mycoplasma gallisepticum* infections in house finches. *Auk,* **118**, 327-333.

524   Hedrick PW (2002) Pathogen resistance and genetic variation at MHC loci. *Evolution,* **56**,

525   1902-1908.

526   Hickman-Davis JM (2002) Role of innate immunity in respiratory mycoplasma infection.

527   *Frontiers in Bioscience,* **7**, D1347-D1355.

528   Hickman-Davis JM, Lindsey JR, Zhu S, Matalon S (1998) Surfactant protein A mediates

529   mycoplasmacidal activity of alveolar macrophages. *American Journal of Physiology-*

530   *Lung Cellular and Molecular Physiology,* **274**, L270-L277.

531   Hickman-Davis JM, Michalek SM, Gibbs-Erwin J, Lindsey JR (1997) Depletion of alveolar

532   macrophages exacerbates respiratory mycoplasmosis in mycoplasma-resistant C57BL

533   mice but not mycoplasma-susceptible C3H mice. *Infection and Immunity,* **65**, 2278-

534   2282.

535   Hochachka WM, Dhondt AA (2000) Density-dependent decline of host abundance resulting

536   from a new infectious disease. *Proceedings of the National Academy of Sciences of*

537   *the United States of America,* **97**, 5303-5306.

538   Hommais F, Pereira S, Acquaviva C, Escobar-Paramo P, Denamur E (2005) Single-

539   nucleotide polymorphism phylotyping of *Escherichia coli. Applied and*

540   *Environmental Microbiology,* **71**, 4784-4792.

541   Iwasaki A, Medzhitov R (2010) Regulation of adaptive immunity by the innate immune

542   system. *Science,* **327**, 291-295.

543   Janeway C (2005) *Immunobiology : the immune system in health and disease* Garland

544   Science, New York :.

545   Janeway CA (1989) Approaching the asymptote - evolution and revolution in immunology.

546   *Cold Spring Harbor Symposia on Quantitative Biology,* **54**, 1-13.

547   Javed M, Frasca S, Cecchini K*, et al.* (2007) Chemokine and cytokine gene expression

548   profiles in chickens inoculated with *Mycoplasma gallisepticum* strains R-low or GT5.

549   *Vaccine,* **25**, 8611-8621.

550   Jenner RG, Young RA (2005) Insights into host responses against pathogens from

551   transcriptional profiling. *Nature Reviews Microbiology,* **3**, 281-294.

552   Kalina M, Blau H, Riklis S, Hoffman V (2000) Modulation of nitric oxide production by lung

553   surfactant in alveolar macrophages. In: *Biology and Pathology of Innate Immunity*

554   *Mechanisms* (eds. Keisari Y, Ofek I), pp. 37-48.

555   Kerr PJ, Best SM (1998) Myxoma virus in rabbits. *Revue Scientifique Et Technique De L*

556   *Office International Des Epizooties,* **17**, 256-268.

557   Lai WC, Bennett M, Pakes SP*, et al.* (1990) Resistance to *Mycoplasma pulmonis* mediated by

558   activated natural-killer-cells. *Journal of Infectious Diseases,* **161**, 1269-1275.

559   Lai WC, Pakes SP, Lu YS, Brayton CF (1987) *Mycoplasma pulmonis* infection augments

560   natural-killer cell-cctivity in mice. *Laboratory Animal Science,* **37**, 299-303.

561   Ledford JG, Pastva AM, Wright JR (2010) Collectins link innate and adaptive immunity in

562   allergic airway disease. *Innate Immunity,* **16**, 183-190.

563   Ley DH (2008) *Mycoplasma gallisepticum* Infection. In: *Diseases of Poultry* (ed. Saif YM),

564   pp. 722-743. Iowa State Press, Ames, U.S.A.

565   Ley DH, Berkhoff, J.E., McLaren, J.M. (1996) Isolation of *Mycoplasma gallisepticum* in

566   house finches with conjunctivitis. *Avian Diseases,* **40**, 480-483.

567 Lips KR, Brem F, Brenes R*, et al.* (2006) Emerging infectious disease and the loss of

568   biodiversity in a Neotropical amphibian community. *Proceedings of the National*

569   *Academy of Sciences of the United States of America,* **103**, 3165-3170.

570 Luttrell MP, Fischer JR, Stallknecht DE, Kleven SH (1996) Field investigation of

571   *Mycoplasma gallisepticum* infections in house finches (*Carpodacus mexicanus*) from

572   Maryland and Georgia. *Avian Diseases.,* **40**, 335-341.

573 Luttrell MP, Stallknecht DE, Fischer JR, Sewell CT, Kleven SH (1998) Natural *Mycoplasma*

574   *gallisepticum* infection in a captive flock of house finches. *Journal of Wildlife*

575   *Diseases,* **34**, 289-296.

576 Manceau M, Domingues VS, Mallarino R, Hoekstra HE (2011) The Developmental Role of

577   Agouti in Color Pattern Evolution. *Science,* **331**, 1062-1065.

578 Markham PF, Glew MD, Browning GF, Whithear KG, Walker ID (1998) Expression of two

579   members of the pMGA gene family of *Mycoplasma gallisepticum* oscillates and is

580   influenced by pMGA-specific antibodies. *Infection and Immunity,* **66**, 2845-2853.

581 Marquis JF, Nantel A, LaCourse R*, et al.* (2008) Fibrotic response as a distinguishing feature

582   of resistance and susceptibility to pulmonary infection with *Mycobacterium*

583   *tuberculosis* in mice. *Infection and Immunity,* **76**, 78-88.

584 Marshall AJ, Miles RJ, Richards L (1995) The Phagocytosis of Mycoplasmas. *Journal of*

585   *Medical Microbiology,* **43**, 239-250.

586 Marshall ID, Fenner F (1958) Studies in the epidemiology of infectious myxomatosis of

587   rabbits. V. Changes in the innate resistance of wild rabbits between 1951 and 1959.

588   *Journal of Hygiene,* **56**, 288-302.

589   Matsuo K, Kuniyasu C, Yamada S, Susumi S, Yamamoto S (1978) Suppression of immuno-

590   responses to *Hemophilus gallinarum* with nonviable *Mycoplasma gallisepticum* in

591   chickens. *Avian Diseases,* **22**, 552-561.

592   Mebius RE, Kraal G (2005) Structure and function of the spleen. *Nature Reviews*

593   *Immunology,* **5**, 606-616.

594   Mohammed J, Frasca S, Cecchini K*, et al.* (2007) Chemokine and cytokine gene expression

595   profiles in chickens inoculated with *Mycoplasma gallisepticum* strains R-low or GT5.

596   *Vaccine,* **25**, 8611-8621.

597   Naylor CJ, Alankari AR, Alafaleq AI, Bradbury JM, Jones RC (1992) Exacerbation of

598   *Mycoplasma gallisepticum* Infection in Turkeys by Rhinotracheitis Virus. *Avian*

599   *Pathology,* **21**, 295-305.

600   Nolan PM, Hill GE, Stoehr AM (1998) Sex, size, and plumage redness predict house finch

601   survival in an epidemic. *Proceedings of the Royal Society of London Series B-*

602   *Biological Sciences,* **265**, 961-965.

603   Noller AC, McEllistrem MC, Stine OC*, et al.* (2003) Multilocus sequence typing reveals a

604   lack of diversity among *Escherichia coli* O157 : H7 isolates that are distinct by

605   pulsed-field gel electrophoresis. *Journal of Clinical Microbiology,* **41**, 675-679.

606   Nordberg J, Arner ESJ (2001) Reactive oxygen species, antioxidants, and the mammalian

607   thioredoxin system. *Free Radical Biology and Medicine,* **31**, 1287-1312.

608   Piertney SB, Oliver MK (2006) The evolutionary ecology of the major histocompatibility

609   complex. *Heredity,* **96**, 7-21.

610   Raida MK, Buchmann K (2008) Development of adaptive immunity in rainbow trout,

611   *Oncorhynchus mykiss* (Walbaum) surviving an infection with *Yersinia ruckeri. Fish*

612   *& Shellfish Immunology,* **25**, 533-541.

613   Razin S, Yogev D, Naot Y (1998) Molecular biology and pathogenicity of mycoplasmas.

614   *Microbiology and Molecular Biology Reviews,* **62**, 1094-1156.

615   Roberts SR, Nolan PM, Hill GE (2001a) Characterization of *Mycoplasma gallisepticum*

616   infection in captive house finches (*Carpodacus mexicanus*) in 1998. *Avian Diseases,*

617   **45**, 70-75.

618   Roberts SR, Nolan PM, Lauerman LH, Li LQ, Hill GE (2001b) Characterization of the

619   mycoplasmal conjunctivitis epizootic in a house finch population in the southeastern

620   USA. *Journal of Wildlife Diseases,* **37**, 82-88.

621   Samstag Y, Eibert SM, Klemke M, Wabnitz GH (2003) Actin cytoskeletal dynamics in T

622   lymphocyte activation and migration. *Journal of Leukocyte Biology,* **73**, 30-48.

623   Sarson AJ, Parvizi P, Lepp D, Quinton M, Sharif S (2008) Transcriptional analysis of host

624   responses to Marek's disease virus infection in genetically resistant and susceptible

625   chickens. *Animal Genetics,* **39**, 232-240.

626   Scheele JS, Marks RE, Boss GR (2007) Signaling by small GTPases in the immune system.

627   *Immunological Reviews,* **218**, 92-101.

628   Shapiro MD, Marks ME, Peichel CL*, et al.* (2004) Genetic and developmental basis of

629   evolutionary pelvic reduction in threespine sticklebacks. *Nature,* **428**, 717-723.

630   Sommer S (2005) The importance of immune gene variability (MHC) in evolutionary

631   ecology and conservation. *Frontiers in Zoology,* **2**, 16.

632   Spurgin LG, Richardson DS (2010) How pathogens drive genetic diversity: MHC,

633   mechanisms and misunderstandings. *Proceedings of the Royal Society B-Biological*

634   *Sciences,* **277**, 979-988.

635   van de Wetering JK, van Golde LMG, Batenburg JJ (2004) Collectins - Players of the innate

636   immune system. *European Journal of Biochemistry,* **271**, 1229-1249.

637   van der Most PJ, de Jong B, Parmentier HK, Verhulst S (2011) Trade-off between growth

638   and immune function: a meta-analysis of selection experiments. *Functional Ecology,*

639   **25**, 74-80.

640   van der Sar AM, Spaink HP, Zakrzewska A, Bitter W, Meijer AH (2009) Specificity of the

641   zebrafish host transcriptome response to acute and chronic mycobacterial infection

642   and the role of innate and adaptive immune components. *Molecular Immunology,* **46**,

643   2317-2332.

644   Wang JB, Nygaard V, Smith-Sorensen B, Hovig E, Myklebost O (2002) MArray: analysing

645   single, replicated or reversed microarray experiments. *Bioinformatics,* **18**, 1139-1140.

646   Wang Z, Farmer K, Hill G, Edwards SV (2006) A cDNA macroarray approach to parasite-

647   induced gene expression in a songbird host: genetic response of House Finches to

648   experimental infection by *Mycoplasma gallisepticum. Molecular Ecology,* **15**, 1263-

649   1273.

650   Whitcomb RF (1983) Culture media for spiroplasmas. In: *Methods in mycoplasmology* (eds.

651   Razin S, Tully JG), pp. 147-158. Academic Press, New York, New York.

652   Wright S (1955) Classification of the Factors of Evolution. *Cold Spring Harbor Symposia on*

653   *Quantitative Biology,* **20**, 16-D24.

654   Yang YH, Speed T (2002) Design issues for cDNA microarray experiments. *Nature Reviews*

655   *Genetics,* **3**, 579-588.

656

657

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 (1st treatment/population *vs*. 2nd 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.

Fig. 1

(A)

(B)

(C)

30