Antibiotic perturbation of gut bacteria does not significantly alter host responses to ocular disease in a songbird species

Chava L. Weitzman[1‡*], Lisa K. Belden[1], Meghan May[2], Marissa M. Langager[1], Rami A. Dalloul[3], Dana M. Hawley[1]

[1] Department of Biological Sciences, Virginia Tech, Blacksburg, VA, USA

[2] Department of Biomedical Sciences, University of New England, Biddeford, ME, USA

[3] Department of Poultry Science, University of Georgia, Athens, GA, USA

‡Current Affiliation: Research Institute for the Environment and Livelihoods, Charles Darwin University, Darwin, NT, Australia

*Corresponding Author:

Chava Weitzman

Research Institute for the Environment and Livelihoods, Charles Darwin University, Darwin, NT, Australia

Email address: weitzman.chava@gmail.com

Abstract

Bacterial communities in and on wild hosts are increasingly appreciated for their importance in host health. Through both direct and indirect interactions, bacteria lining vertebrate gut mucosa provide hosts protection against infectious pathogens, sometimes even in distal body regions through immune regulation. In house finches (*Haemorhous mexicanus*), the bacterial pathogen *Mycoplasma gallisepticum* (MG) causes conjunctivitis, with ocular inflammation mediated by pro- and anti-inflammatory cytokines and infection triggering MG-specific antibodies. Here, we tested the role of gut bacteria in host responses to MG by using oral antibiotics to perturb bacteria in the gut of captive house finches prior to experimental inoculation with MG. We found no clear support for an impact of gut bacterial disruption on conjunctival pathology, MG load, or plasma antibody levels. However, there was a non-significant trend for birds with intact gut communities to have greater conjunctival pathology, suggesting a possible impact of gut bacteria on pro-inflammatory cytokine stimulation. Using 16S bacterial rRNA amplicon sequencing, we found dramatic differences in cloacal bacterial community composition between captive, wild-caught house finches in our experiment and free-living finches from the same population, with lower bacterial richness and core communities composed of fewer genera in captive finches. We hypothesize that captivity may have affected the strength of results in this experiment, necessitating further study with this consideration. The abundance of anthropogenic impacts on wildlife and their bacterial communities, alongside the emergence and spread of infectious diseases, highlights the importance of studies addressing the role of commensal bacteria in health and disease, and the consequences of gut bacterial shifts on wild hosts.

Introduction

Symbiotic bacterial communities interact with invading pathogens in diverse ways and are increasingly recognized as important players in host health and disease (Daskin & Alford, 2012; Boon et al., 2014; Oliver, Smith & Russell, 2014; Becker et al., 2015). Beyond direct interactions influencing pathogen invasion and growth, commensal bacteria aid in host immune system development and regulate immune responses throughout life (Honda & Littman, 2012; Kabat, Srinivasan & Maloy, 2014; Lazar et al., 2018; Broom & Kogut, 2018; Kogut, Lee & Santin, 2020), indirectly affecting the strength and efficiency of hosts' responses to pathogens. Bacteria along the mucosal surfaces of the gastro-intestinal tract (hereafter "gut"), in particular, provide hosts with diverse types of protection from infection, including serving as a physical barrier to pathogens on mucosal surfaces, and indirectly regulating host immunity in ways that often facilitate pathogen clearance (Khosravi & Mazmanian, 2013). On the other hand, gut bacteria can have complicated effects on host inflammatory immune responses, both in the gut and other tissues (Ichinohe et al., 2011; Hooper, Littman & Macpherson, 2012; Wilks & Golovkina, 2012; Shukla et al., 2017; Lazar et al., 2018; Kogut, Lee & Santin, 2020), making the effects of commensal gut bacteria on host responses to infections difficult to predict. As anthropogenic impacts on wildlife and their bacterial communities increase in extent, via antibiotics and other stressors (Kelly et al., 2014; Teyssier et al., 2018; Trevelline et al., 2019; Kraemer, Ramachandran & Perron, 2019; Lavrinienko et al., 2021), a better understanding of the importance of bacterial communities in wildlife hosts is imperative to prepare for, and predict, effects of emerging wildlife diseases.

While the importance of gut bacteria in health and disease has been established (Honda & Littman, 2012), the majority of studies focus on human health and mammalian model systems,

with studies of wild and captive non-model species largely limited to characterizing gut communities within and among species (e.g. Yuan et al., 2015; Cheng et al., 2015; Kohl et al., 2017; Teyssier et al., 2018; Song et al., 2020). Such studies broadly show that gut bacterial communities are influenced by both endogenous and exogenous factors and are maintained by interactions among hosts and between hosts and their environment (Harris, Roode & Gerardo, 2019; Koskella & Bergelson, 2020; Sarkar et al., 2020). For example, physiological stressors (Vlčková et al., 2018; Keohane et al., 2019), disease elsewhere in the body (Vieira & Pretorius, 2010; Wang et al., 2014), warming environmental temperatures (Bestion et al., 2017), and urbanization (Teyssier et al., 2018; Murray et al., 2020) can all influence gut microbial communities among individuals and populations. While the role of such gut microbial variation in wildlife health remains largely unknown (but see Davidson et al., 2021; Worsley et al., 2021), the growing number of factors shown to influence gut microbial composition in wild systems indicates that the consequences of such variation for wildlife health requires broader attention. These interactions are ideal for studying with controlled captive experiments to identify factors in natural systems that may require greater consideration in their effects on wildlife health. As such, captive studies of gut bacterial function in wild-caught animals can provide an important intermediate link between studies documenting gut microbial variation in free-living animals and experimental studies of gut bacteria and host health in lab animal models.

Few studies have directly examined the potential for gut bacteria in wild animals to influence host responses to pathogens, though studies of lab animal models suggest that such interactions are likely. Experiments in mice find that both innate and adaptive immune responses to respiratory viral infections are triggered by resident gut microbes (Ichinohe et al., 2011; Abt et al., 2012). Specifically, in response to experimental infection, mice with intact gut communities

had shorter infection times, but increased inflammatory and antibody responses, compared with antibiotic-treated mice. In contrast, germ-free laboratory mice inoculated with microbiota from wild mice show reduced inflammation from influenza virus and increased survival (Rosshart et al., 2017). Similarly, mice given oral antibiotics to knock down gut microbes generally had greater inflammatory responses to *Streptococcus pneumoniae* infection than those with intact gut microbiomes (Schuijt et al., 2016). These results provide support for a key role of gut microbes mediating host inflammatory responses to pathogens. Importantly, there is evidence that diseases characterized by inflammatory responses may be particularly affected by gut bacteria, while gut microbes can be less important for non-inflammatory responses to infection. For example, pathogens that do not trigger inflammasome-dependent cytokine responses (e.g. *Legionella pneumophila*) in hosts were not affected by knocking down the resident gut bacteria (Ichinohe et al., 2011). These experiments highlight the varied roles of gut communities in host disease, though a broader understanding of these interactions in non-mammalian and non-model systems is necessary.

In wild house finches (*Haemorhous mexicanus*), the ocular pathogen *Mycoplasma gallisepticum* (MG), which causes mycoplasmal conjunctivitis, has spread across much of the host's distribution in the continental United States (Ley et al., 2016). Disease outbreaks in finches are associated with reduced host fitness and resulting population declines (Hochachka & Dhondt, 2000; Faustino et al., 2004). The degree of inflammation of the conjunctiva during infection is mediated by both pro- and anti-inflammatory cytokines (Vinkler et al., 2018). Because gut bacteria are mediators of immune responses in diverse vertebrates (Rosshart et al., 2017; Grond et al., 2018), they may be important during ocular infection in this system. Further, the conjunctival inflammation that house finches experience during infection appears to predict

116 their likelihood of mortality from infection, which occurs largely via predation (Adelman, Mayer
117 & Hawley, 2017); thus, understanding what factors drive variation in conjunctival inflammation,
118 such as the gut bacterial community, is key for predicting the fitness impacts of this pathogen on
119 host populations.

120 In this experiment, we tested the role of resident gut bacteria on disease in peripheral
121 ocular tissues during mycoplasmal infection. We disrupted the gut bacteria through
122 administration of oral antibiotics and then experimentally inoculated birds with MG to test the
123 hypothesis that disrupted gut bacterial communities would affect disease caused by MG. We
124 predicted that if gut bacteria stimulate pro-inflammatory cytokine production in this system, then
125 antibiotic-treated birds would show less severe conjunctival inflammation, given documented
126 associations between conjunctivitis severity and pro-inflammatory cytokine expression (Vinkler
127 et al., 2018). Alongside reduced pathology, we expected to find reduced MG-specific antibody
128 production, higher mycoplasmal loads, and longer disease, as detected in other systems (Abt et
129 al., 2012). Alternatively, because pathology is coincident with mycoplasmal tissue damage, we
130 considered that we could find increased pathology in antibiotics-treated birds. To address the
131 hypothesis that we could detect shifts in gut bacteria due to antibiotics administration, we used
132 amplicon sequencing of cloacal swabs as a proxy for mucosal bacteria in the gut to avoid
133 destructive sampling of hosts that we were actively monitoring for conjunctival disease
134 outcomes. Lastly, to put our captive experiment on this wild-caught species into context, we
135 compared cloacal swabs from our experimental birds to free-living birds to assess the differences
136 in bacterial communities in captive versus wild house finches.

137

138 ## Materials & Methods

Bird Capture and Housing

Hatch-year house finches (n = 50) were wild-caught in June–July 2020 in Blacksburg, Montgomery County, Virginia. Upon capture, finches were housed singly or in pairs and subjected to a two week quarantine period, during which time we verified that no bird exhibited clinical signs of conjunctivitis. After two weeks, we also tested for MG-specific antibodies with enzyme-linked immunosorbent assay (ELISA; Hawley et al., 2011), and no seropositive birds were included in the present study. To minimize mortality due to coccidiosis, all birds were treated with Endocox (toltrazuril) in their water (1.29 g/L for three days every one to four weeks). Endocox treatment should not have directly affected the gut bacteria (Maurice et al., 2015), and treatment ceased 18 days before MG inoculation. Birds were single-housed 13 days prior to inoculation, at the end of September 2020 (Fig. 1), and were provided a constant 12:12 light-dark cycle and food and water *ad libitum.* Birds were captured under VDGIF (066646) and USFWS (MB158404-0) permits. Experimental procedures were approved by Virginia Tech's Institutional Animal Care and Use Committee (BIOL-18-144).

Experimental Design

Experimental birds were randomly assigned to oral antibiotics and MG treatments in a 2 × 2 factorial design, with as close to 50:50 sex ratio as possible per treatment group. To perturb the gut bacteria with antibiotics, we administered a mixture of amoxicillin and metronidazole in the birds' water (available *ad libitum*), at final concentrations of 1000 mg/L and 200 mg/L respectively, for seven days prior to MG inoculation (Dorrestein, 2009, n = 25 birds, Fig. 1, Table 1). Water (control) and antibiotics were refreshed daily. The length of these broad-spectrum antibiotics administration was chosen based on studies in chickens to cause large enough changes to affect immune responses (Wise & Siragusa, 2007; Pélissier et al., 2010;

Pallav et al., 2014; Schokker et al., 2017; Connelly et al., 2018; Xiong et al., 2018). Though these antibiotics may be spread systemically through the bird, they both have short half-lives in other birds (≤ 12 hrs in poultry; Anadón et al., 1996; Cybulski et al., 1996), and we ceased antibiotics treatment prior to MG inoculation. Further, any residual antibiotics present in birds would not affect MG itself as they mainly affect bacteria with cell walls (amoxicillin) and anaerobic bacteria (metronidazole) (Anadón et al., 1996; Bébéar, Pereyre & Peuchant, 2011; Ceruelos et al., 2019). On MG inoculation day, we administered 70 µL of Frey's media (control, Table 1) or MG diluted in Frey's media (VA1994 isolate, 7994-1 6 P 9/17/2018) to a concentration of 3×10^4 color changing units/mL (CCU/mL) by droplet instillation onto the conjunctivae.

We focused our data collection on following disease in birds throughout infection, making us unable to measure cytokine expression in this study, which requires destructive sampling (Vinkler et al., 2018). Birds were monitored for pathology and MG load periodically throughout infection (Fig. 1). We scored the degree of pathology for each conjunctiva on a scale of 0–3 and summed the values between the two eyes for a given sampling day (Sydenstricker et al., 2005). No experimental birds had any pathology prior to MG inoculation. To measure MG loads, cotton-tipped swabs were lubricated with tryptose phosphate broth (TPB) before gently swabbing each conjunctiva and wringing out the swab in a collection tube of 300 µL TPB. Both conjunctival swab samples per bird were combined into a single TPB tube. Both eye scoring and swabbing were done by a single individual (CLW), blind to treatment.

Measuring Pathogen Load

184 We extracted DNA from a subset of ocular swab samples from MG-treated birds on post-
185 inoculation days –1, 3, 7, and 13 (n = 8–10 per treatment per day; Table S1) using the Qiagen
186 DNeasy 96 Blood and Tissue Kit (Qiagen, Valencia, CA) to examine differences between the
187 treatment groups in MG loads early in infection. We also extracted samples from sham MG birds
188 on day 3 to ensure that our sham controls were MG-free, because day –1 samples from these
189 birds were used for a separate study. We included four extraction controls (extraction reagents
190 with no sample) on the DNA extraction plate interspersed among our samples. MG load was
191 measured with a probe-based quantitative polymerase chain reaction (qPCR) amplifying the
192 MGC2 gene, as previously described (Grodio et al., 2008; Leon & Hawley, 2017). With every
193 MGC2 qPCR plate run we included a standard curve of eight plasmid concentrations (10^1 to 10^8
194 copies) in triplicate.
195 We verified with ocular swab samples from day –1 that birds in MG inoculated groups
196 did not begin the experiment with active MG infections; those samples were negative for MG via
197 qPCR. Some of the sham control bird samples from day 3 were positive for low levels of MG via
198 qPCR (max of $10^{1.66}$ copies; Fig. 2b), but the lowest value from a MG-inoculated bird on that
199 post-infection day ($10^{2.55}$ copies) was 8-times greater than the highest MG sham control sample.
200 These positive MG sham control samples were likely contaminated during DNA extraction,
201 supported by the absence of pathology for any MG-control birds throughout the experiment. The
202 qPCR reaction we use is very sensitive to low levels of contamination, which have been detected
203 previously in this system (Leon & Hawley, 2017). Because treatment groups were randomized
204 among the extraction plate, low-level contamination is not likely to affect our results. None of
205 the extraction controls tested positive for MG from qPCR.
206

Bacterial Community Characterization

In birds and many other vertebrates, the cloaca is the terminal opening for the digestive, urinary, and reproductive tracts, making cloacal swabbing a minimally invasive method standardly used in avian studies for collecting microbial communities (Klomp et al., 2008; Escallón et al., 2017). We used amplicon sequencing of cloacal swab samples to address the hypothesis that we could detect shifts in the gut bacterial communities due to antibiotics with cloacal swab data, as well as the hypothesis that cloacal bacteria in captive house finches are representative of those in wild house finches.

On day –1 post-inoculation (day 6 of antibiotics treatment) and day 7 post-inoculation (Fig. 1), we gently inserted a sterile swab (PurFlock Ultra®, Puritan, Guilford, Maine) ~4 mm into the cloaca and rotated for 5 seconds. Swabs were placed in sterilized 1.5 mL microcentrifuge tubes on ice after collection and frozen at –80 ˚C until DNA extraction. We extracted DNA from 49 cloacal swab samples (Table 1) with the Qiagen DNeasy Blood and Tissue Kit protocol for Gram-positive bacteria. We focused DNA extraction on detecting effects of antibiotics and avoiding confounding effects of MG infection, extracting both sample dates for all 16 MG control birds and only day –1 samples from birds subsequently given MG. To compare cloacal bacterial communities from captive birds with those in the wild from the same population, we also extracted DNA (using identical methods) from 16 cloacal swab samples collected from wild house finches in October–November 2020 in Blacksburg, VA.

Unlike the extraction methods detailed above for ocular swab samples (96-well extraction), our DNA extractions of cloacal samples were conducted in a biosafety cabinet with single-tube extractions to minimize contamination into and among the samples. Alongside the cloacal swab extractions, we extracted DNA from environmental controls (n = 4; exposing a

swab to the air) and extraction controls (n = 5), and conducted library prep on n = 6 of these control samples, but did not include them in the sequence run. From Qubit analysis, average ± SD ng/μL of library prepped cloacal samples and controls were 16.7 ± 10.4 and 2.2 ± 1.0 respectively. Thus, we are confident that the bacterial communities described here largely constitute those in the cloaca and not from outside sources.

For cloacal swab bacterial community assessment, we amplified the V4 region of bacterial 16S rRNA gene with the 515F/806R primers (Caporaso et al., 2012) as previously described (Thomason et al., 2017; Hernandez et al., 2020; Weitzman et al., 2021), and amplicons were sequenced using a 250bp single-end strategy on an Illumina MiSeq at the Dana Farber Cancer Center of Harvard University. Forward reads were demultiplexed in QIIME2 (Bolyen et al., 2019) and processed with the DADA2 package with R version 4.0.2 in RStudio version 1.3.1093 (R Development Core Team, 2015; Callahan et al., 2016; RStudio Team, 2020). With the filterAndTrim function, we kept reads with a minimum length of 250 bases and 5 maximum expected errors. After inferring amplicon sequence variants (ASVs), we removed chimeras, assigned taxonomy with the Silva v132 database using the assignTaxonomy command, and removed non-bacterial, mitochondrial, and chloroplast reads. After inspection of rarefaction curves, we rarefied samples to 5000 reads for richness and alpha diversity analyses and kept unrarefied data from samples with a minimum of 1000 reads for beta diversity analyses and analysis of differential abundance. Beta diversity metrics were calculated from data transformed to proportional reads (on a per sample basis, reads per ASV divided by total reads; McKnight et al., 2019). We used QIIME2 to determine ASV richness, Faith's phylogenetic diversity, Bray-Curtis and Jaccard distances, and weighted and unweighted UniFrac distances. UniFrac reflects phylogenetic-based distances, with unweighted UniFrac distances based off presence-absence

253 data and weighted UniFrac distances incorporating relative abundance. From these diversity
254 metrics, we calculated the change in richness and phylogenetic diversity per bird where
255 applicable (day 7 post-inoculation – day –1 pre-inoculation) and also extracted pairwise distance
256 values of samples between day –1 and day 7 post-inoculation per bird. For analyses of
257 differential abundance, we collapsed the ASV table to genus level.
258
259 *Statistical Analyses*
260 Statistical analyses were run with R version 4.0.2 in RStudio version 1.3.1093 (R
261 Development Core Team, 2015; RStudio Team, 2020). The full models for pathology, MG load,
262 and probability of infection included antibiotics treatment, post-inoculation day, their interaction,
263 sex, and a random variable of bird ID. We used model simplification to arrive at final models,
264 removing the interaction term or covariates (day, sex) when $p > 0.1$ from Wald's tests in the car
265 package (Fox & Weisberg, 2019). We used linear mixed-effects models (LMM) with the lme4
266 package (Bates et al., 2015), analyzing pathology data from MG-inoculated birds only, to test the
267 hypothesis that oral antibiotics influenced the degree of house finch conjunctival pathology after
268 inoculation with MG. To fit assumptions of normality in analyses, we analyzed pathology data as
269 log(sum eye score + 1). We similarly analyzed MG load, as $\log_{10}(\text{load} + 1)$, with LMM to test
270 the hypothesis that oral antibiotics treatment influenced MG growth. Here we modeled post-
271 inoculation day as an ordinal variable because MG load was only measured on three post-
272 inoculation days. We additionally analyzed whether the probability of infection (Y/N) differed
273 based on antibiotics treatment, defining successful infection as a conjunctival MG load > $10^{3.1}$
274 copies (Adelman et al., 2015; Leon & Hawley, 2017). Because a subset of samples were
275 randomly extracted for each post-inoculation day, we qualified each MG load as "infected" or

not and analyzed probability of infection with a binomial generalized linear model with a probit link, with day as an ordinal variable as above.

We used 16S rRNA amplicon sequences from cloacal swabs to address two additional hypotheses: oral antibiotics affect cloacal bacterial communities, but communities begin to return to their initial composition one week after antibiotics treatment ends; and cloacal bacteria in captive house finches are similar to, and representative of, those in wild house finches.

To assess the effects of antibiotics on cloacal bacterial communities, we subset the alpha and beta diversity metrics, and their changes over time, to only include captive birds in the experiment. We made three sets of comparisons with these data: differences between communities on day –1 in birds that were given antibiotics and non-treated birds; changes in communities within birds from day –1 to day 7; and differences between communities on day 7 in antibiotics and non-treated control birds. We analyzed log-transformed values of ASV richness and Faith's phylogenetic diversity with LMM, determining p-values with Wald's tests. Analyses included predictor variables of antibiotics treatment, post-inoculation day (categorical with two sampling days), their interaction, sex, and the random variable of bird ID. We assessed differences in bacterial community structure based on the same predictor variables with permutational analysis of variance (PERMANOVA) with a block design (bird ID as random variable) and analysis of beta dispersion in the vegan package (Oksanen et al., 2018), and visualized community structure differences with principal coordinates analysis in the ape package (PCoA; Paradis & Schliep, 2019). ANOVAs were used to compare changes in richness and phylogenetic diversity, and pairwise beta diversity distances (distance value within a bird between day –1 and day 7), between antibiotics treatment groups. Though we initially included

sex as a covariate in each of these analyses, it was never significant and was removed for final results.

We then used genus-level data to detect differentially abundant taxa between antibiotics treatments and sampling days with ALDEx2 (Fernandes et al., 2013, 2014) in QIIME2, with genera present in at least 10% of the samples included in each analysis. Genera were considered differentially abundant when Wilcoxon rank test p-value was below 0.05 with a Benjamini-Hochberg correction (Benjamini & Hochberg, 1995). ALDEx2 can only perform pairwise comparisons between two groups, so we subset and analyzed our data with this consideration, comparing relative abundances of genera between: antibiotics and non-treated birds on day –1 (antibiotics n = 12, control n = 19), day –1 (n = 12) and day 7 (n = 8) in birds given antibiotics, and birds given antibiotics compared with non-treated birds on day 7 (n = 8 each).

To detect whether cloacal communities of captive finches are representative of those of wild birds, we subset the cloacal swab data to include samples from non-antibiotics-treated birds from the experiment on day –1 and samples from wild birds. Thus, no birds in this had received oral antibiotics. We compared log-transformed richness and phylogenetic diversity between these groups (captive versus wild finches) with ANOVAs. We also used PERMANOVA and analysis of beta dispersion to compare beta distances, as well as ALDEx2 to detect differentially abundant genera between these two groups of samples. Finally, we determined the overlap of core genera (present in >85% of samples per group) between these groups.

Results

Infection and Disease

Inoculation with MG resulted in varied host responses, with all but three inoculated birds exhibiting some visible conjunctival pathology during the experiment. The severity of pathology did not differ based on pre-inoculation oral antibiotics treatment (LMM, estimate ± SD = –0.19 ± 0.12, χ^2= 2.35, p = 0.1); covariates of post-inoculation day (and its interaction with antibiotics treatment) and sex were removed from the final model because they were not significant. Although not significant, average differences in pathology followed our predictions, with a lower average degree of conjunctival inflammation in hosts given antibiotics treatment prior to infection compared to controls, particularly early in infection (Fig. 2a).

MG load differed by post-inoculation day, but not by antibiotics treatment (LMM, day: χ^2 = 24.40, $p < 0.0001$; antibiotics treatment: estimate ± SD = –0.14 ± 0.26, χ^2 = 0.27, $p > 0.5$; Fig. 2b). We also assessed the probability of being infected (binomial Y/N) on the post-inoculation days for which we had MG load data, and similarly found that the probability of being infected differed significantly among post-inoculation days, but not based on antibiotics treatment (day: χ^2 = 11.75, p = 0.003; antibiotics treatment: estimate ± SD = 0.08 ± 0.39, χ^2 = 0.04, $p > 0.5$). Finally, we did not find any significant effects of treatment on plasma antibody levels in response to experimental infection (Supplemental Materials).

Cloacal Bacteria in Captive House Finches

In the total 65 cloacal swab samples (including samples from 16 wild birds), we detected 9,460 bacterial ASVs from 1,951,109 total bacterial reads (102–116,421 reads per sample, mean = 30,019 ± 31,073). We removed two samples with fewer than 1,000 reads (one sample from each: day –1 MG treatment, day –1 both antibiotics treatment+MG). Rarefaction removed an additional 12 samples from alpha diversity analyses (Fig. 3). The cloacal communities in our

343 captive house finches at the beginning of the experiment were largely comprised of
344 Proteobacteria (e.g. *Pseudomonas*, *Sphingomonas*, *Janthinobacterium*) and Firmicutes (e.g.
345 *Candidatus Arthromitus*, *Staphylococcus*, *Bacillus*), followed by Actinobacteria, and Tenericutes
346 (Fig. S1).

347 We did not detect strong, immediate effects of antibiotics on cloacal bacterial
348 communities. At day –1 (the final day of oral antibiotics), diversity metrics were largely
349 comparable for non-treated and antibiotics-treated birds (Fig. 3ab). However, antibiotics-treated
350 birds appeared to have less variable bacterial diversity than did non-treated birds at day –1, just
351 prior to MG inoculation, though this pattern reversed on day 7 post-inoculation (Fig. 3ab).
352 Overall, there was no significant effect of antibiotics treatment on bacterial richness (estimate ±
353 SD = 0.26 ± 0.32, $\chi^2 = 0.68$, $p = 0.4$; Fig. 3a), phylogenetic diversity (estimate ± SD = 0.18 ±
354 0.24, $\chi^2 = 0.56$, $p = 0.5$; Fig. 3b), unweighted UniFrac distance (sum of squares = 0.25, $F_{1,45}$ =
355 0.98, $R^2 = 2.1\%$, $p > 0.5$; Fig. S2) or weighted UniFrac distance (sum of squares = 0.29, $F_{1,45}$ =
356 2.12, $R^2 = 4.5\%$, $p > 0.5$, Fig. S2). The difference in community structure based on antibiotics
357 treatment was not explained by differing dispersion (unweighted UniFrac: sum of squares =
358 0.003, $F_{1,45} = 1.27$, $p = 0.3$; weighted UniFrac: sum of squares = 0.03, $F_{1,45} = 1.94$, $p = 0.2$). Beta
359 diversity based on Bray-Curtis and Jaccard distances were similarly not affected by antibiotics
360 treatment (Table S2, Fig. S2). Post-inoculation day, the interaction between day and antibiotics
361 treatment, and host sex were not significant in any comparisons of bacterial communities and
362 were removed from all analyses.

363 When examining within-individual changes in diversity metrics from day –1 to day 7
364 post-inoculation, antibiotics treatment significantly predicted paired weighted UniFrac distances
365 (sum of squares = 0.06, $F_{1,14} = 4.73$, $p = 0.047$; Fig. 3e), with results indicating that samples from

366 birds given antibiotics were more similar between the sampling days than control samples from
367 birds that were not treated. However, we found no effect of antibiotics treatment on within-
368 individual changes over time with respect to most other diversity metrics (Fig. 3; richness: $F_{1,7}$ =
369 0.004, p = 1; phylogenetic diversity: $F_{1,7}$ = 0.007, p = 0.9; paired unweighted UniFrac: $F_{1,14}$ =
370 0.29, p = 0.6; paired Bray-Curtis and Jaccard results in Table S2). Antibiotics-treated birds
371 showed a nonsignificant decline in richness and phylogenetic diversity over time, which could
372 represent a delayed effect of antibiotics, though samples from birds not given antibiotics were
373 highly variable.
374 We used ALDEx2 to detect differentially abundant genera in cloacal bacterial
375 communities between antibiotics treatments and sampling days, but found no differences
376 between the birds given antibiotics and non-treated control birds on day –1 or day 7, and no
377 differences in antibiotics-treated birds between day –1 and day 7.
378
379 *Captive vs Wild House Finch Bacteria*
380 Cloacal samples from captive birds (in the lab 10+ weeks prior to the experiment) that
381 did not receive oral antibiotics had significantly different cloacal bacterial communities
382 compared with birds sampled in the wild at the same time of year (Table S3). Importantly, wild
383 samples had higher ASV richness, and the communities differed in community structure (Table
384 S3, Fig. S3, S4). In ADLEx2 analyses, we found 76 genera (out of 621, 12.2%) to be
385 differentially abundant in cloacal communities between untreated captive birds (day –1) and wild
386 samples (Table S4), most of which (n = 62) were more abundant in wild bird samples. We
387 further found that samples from birds in the lab had simpler core bacterial communities, with
388 fewer prevalent genera than cloacal samples from wild birds (Fig. S5).

Discussion

Experimental and observational studies are increasingly finding that infectious disease outcomes in hosts are affected by resident bacterial communities (Hooper, Littman & Macpherson, 2012; Weyrich et al., 2014; Walke & Belden, 2016; Rosshart et al., 2017). We used oral antibiotics and experimental MG inoculation to quantify indirect effects of gut bacteria on ocular disease in house finches. Though we did not find treatment of the gut bacterial microbiome with antibiotics to significantly affect MG pathology, pathogen load, or antibody responses, antibiotics-treated birds had slightly lower average pathology than did control birds early in infection, though it was not a great enough difference to significantly affect the treatment-by-day interaction in analyses. Further, we detected stark differences in cloacal bacterial communities between captive house finches and those in the wild. We focus our discussion of these experimental results through the lens of bacterial communities in our birds.

Gut Bacteria and Ocular Disease

As in other systems (Bornbusch et al., 2021), we expected to find long-term effects of gut bacterial disruption on immune function and consequently disease response. In this experiment, oral antibiotics prior to infection did not strongly affect ocular pathology after inoculation with a high dose of MG, though the data show a slight trend of antibiotics treatment reducing the average degree of pathology early in infection. Though not significant, an average reduction in early ocular inflammation could indicate that some gut bacteria affected by antibiotics treatment in our study may stimulate the inflammatory immune response. House finches experience an influx of local and systemic pro-inflammatory cytokine production (IL-1β, IFN-γ, TNF-α) early

412 in infection with MG (Adelman et al., 2013; Vinkler et al., 2018), and individual variation in
413 conjunctivitis is significantly predicted by individual variation in local IL-1β expression. In this
414 experiment, we were unable to measure cytokine expression, which requires destructive
415 sampling and thus precludes the ability to track infection outcomes. However, an experimental
416 study in mice found that antibiotics to knock down gut bacteria resulted in decreased
417 inflammatory cytokine production and decreased clinical signs of experimental autoimmune
418 uveitis, a non-infectious ocular disease (Nakamura et al., 2016). Similarly, the gut microbiota in
419 mice has been shown to induce inflammatory immune responses to respiratory infection
420 (Ichinohe et al., 2011), indicating that gut microbiota can stimulate inflammatory responses at
421 diverse mucosal surfaces.
422 In our birds, the reduced average inflammation after gut bacterial perturbation was
423 negligible (~0.5 pathology score reduction), which, even if statistically significant, would not
424 likely result in dramatic differences in the bird's vision and ability to find food or evade
425 predators. However, our characterization of cloacal communities indicates that the bacteria in
426 captive house finches may not have been representative of those in wild birds, having lost many
427 bacteria that were prevalent in wild birds. Consequently, gut bacteria may have greater
428 importance for disease outcomes in wild birds than their captive counterparts. Alternatively, gut
429 bacteria in house finches may not play an important role in inflammatory responses in peripheral
430 tissues. However, the close association between gut bacteria and the bursa of Fabricius, the site
431 of B cell development in birds located within the intestinal tract, suggests that intestinal bacteria
432 should have immunomodulatory effects, particularly in the context of cytokine-mediated
433 inflammation (reviewed in Kohl, 2012). As a nascent field, we cannot compare our results with
434 others from songbirds, though the field is gaining traction in human and mouse models,

particularly regarding nonpathogenic ocular diseases. For example, in addition to the mouse experiment noted above (Nakamura et al., 2016), an observational study in people with non-pathogenic uveitis found shifts in gut communities, including decreased diversity, compared with healthy subjects (Kalyana Chakravarthy et al., 2018). Overall, while we did not find statistical support for effects of gut bacteria in altering host responses to ocular disease in house finches here, studies of local and systemic cytokine responses may still be warranted, particularly early in infection when differences in inflammatory immune responses appear important in driving host disease outcomes (Adelman et al., 2013).

Describing and Disturbing Gut Bacteria

The absence of detectable effects of gut bacteria on host responses in our study may have at least partly resulted from limitations in our ability to meaningfully perturb the gut microbiome in captive house finches. Through amplicon sequencing, we detected dramatic differences between the cloacal bacteria of our captive house finches and those sampled in the wild at the time of our study. Notably, wild finches had greater cloacal bacterial richness, with greater relative abundance of many genera when compared with captive finches (Figure S3). Captivity can affect host-associated bacterial communities due to changes in environment, diet, and stressors (Bailey et al., 2010; Becker et al., 2014; Clayton et al., 2016; Bates et al., 2019), and captivity was found to affect bacterial communities in other birds (e.g. Oliveira et al., 2020; San Juan, Castro & Dhami, 2021). However, one study suggests that birds in particular tend to have large quantities of transient bacteria (Song et al., 2020), which may account for a portion of the differences we found between our captive and wild samples. Conducting this experiment on wild-caught birds ensured that their starting communities at least partially represented those of

458 wild birds; however, months in captivity may have affected the bacteria enough to affect our
459 ability to fully test their importance in mycoplasmal conjunctivitis in wild house finches.
460 In the limited laboratory environment, it is not surprising that microbial diversity was
461 significantly lower when compared to birds in the wild. Some captive birds appeared to lose
462 hundreds of bacterial ASVs between the sampling days, possibly because the communities were
463 still shifting as a result of being single-housed. In another study, captive ptarmigans not only had
464 different cecal bacterial community composition, but also a lower total number of bacterial cells
465 per cecal weight when compared with wild ptarmigans (Salgado-Flores et al., 2019). In
466 laboratory mice, microbiome-mediated immune protection was restored when germ-free mice
467 were recolonized with wild, but not lab mouse gut communities (Rosshart et al., 2017).
468 Interestingly, a large comparative study found that birds tend to have lower bacterial counts in
469 their feces, suggesting overall lower resident bacterial microbiome biomass (Song et al., 2020).
470 For birds with low gut bacterial biomass, it is possible that few taxa provide the majority of the
471 benefit of gut communities. Future studies on house finches with more natural gut communities
472 may better reveal effects of gut microbes on host responses.
473 The changes in gut bacteria due to captivity may not have just affected its protective role;
474 they may have also hampered our ability to use antibiotics to thoroughly test the bacteria's role
475 in protection, if captive birds harbor a less abundant and less complex microbiome. From
476 amplicon sequencing of cloacal bacteria, we detected subtle but not strong differences in
477 community structure or composition based on antibiotics treatment. Interestingly, we found that
478 birds given antibiotics had more similar communities over an eight-day period (day –1 to day 7)
479 than those that did not receive antibiotics, based on weighted UniFrac distances. While data from
480 cloacal samples as a proxy for gut bacteria should be interpreted with caution, this suggests that

changes in cloacal bacteria due to the antibiotics prior to inoculation may have lasted well beyond inoculation day. Strikingly, however, we found no indication of reduced cloacal bacterial diversity in antibiotic-treated relative to control birds, suggesting that oral antibiotics may not have had strong effects on gut bacterial diversity in captive house finches. Another study in chickens, which used antibiotic administration to investigate the importance of gut bacteria on immune development, also only found minimal detectable changes in amplicon sequencing of luminal bacteria (Schokker et al., 2017). On the other hand, our ability to detect differences in bacterial diversity was limited by our sampling procedures, which were more likely to detect luminal bacteria than those with a close association with gut mucosal surfaces. Thus, we may have missed important effects of antibiotics on mucosal bacteria, which were found to be more affected by oral antibiotics than luminal bacteria in another songbird system (Kohl et al., 2018). Further, cloacal swabbing picks up bacteria leaving the gut, but is not always a reliable method of measuring bacteria higher in the intestinal tract, including the colon, cecum, and ileum (Videvall et al., 2018). However, preliminary data from cloacal and colon swab samples from captive house finches show that cloacal swabs do loosely represent communities in the lower gastro-intestinal tract (see Supplemental Materials). Further study using destructive sampling should examine effects of antibiotics on gut communities directly, which may shed more light on any changes in gut microbes that resulted from antibiotics treatment and their potential role in host responses to disease.

Conclusions

While we did not find strong evidence for effects of gut bacteria on mycoplasmal conjunctivitis in captive house finches, the nonsignificant trend of decreased pathology after

antibiotics indicates that further experiments are warranted. Experiments with house finches sooner after capture, and with the addition of cytokine expression data collection from several tissues including the gastro-intestinal tract, may more definitively reveal whether and how gut bacteria play a role in the individual- and population-level effects of MG found in wild populations.

Acknowledgements

Thank you to Sara Teemer, the rest of the Hawley lab, and Emma Bradford for their help in the field and lab. We thank three anonymous reviewers for their helpful comments to improve the quality of this paper.

References

Abt MC, Osborne LC, Monticelli LA, Doering TA, Alenghat T, Sonnenberg GF, Paley MA, Antenus M, Williams KL, Erikson J. 2012. Commensal bacteria calibrate the activation threshold of innate antiviral immunity. *Immunity* 37:158–170. DOI: 10.1016/j.immuni.2012.04.011.

Adelman JS, Kirkpatrick L, Grodio JL, Hawley DM. 2013. House finch populations differ in early inflammatory signaling and pathogen tolerance at the peak of *Mycoplasma gallisepticum* infection. *The American Naturalist* 181:674–689. DOI: 10.1086/670024.

Adelman JS, Mayer C, Hawley DM. 2017. Infection reduces anti-predator behaviors in house finches. *Journal of Avian Biology* 48:519–528. DOI: 10.1111/jav.01058.

525 Adelman JS, Moyers SC, Farine DR, Hawley DM. 2015. Feeder use predicts both acquisition
526 and transmission of a contagious pathogen in a North American songbird. *Proceedings of*
527 *the Royal Society B: Biological Sciences* 282:20151429. DOI: 10.1098/rspb.2015.1429.
528 Anadón A, Martinez-Larrañaga MR, Diaz MJ, Bringas P, Fernandez MC, Martinez MA,
529 Fernandez-Cruz ML. 1996. Pharmacokinetics of amoxicillin in broiler chickens. *Avian*
530 *Pathology* 25:449–458. DOI: 10.1080/03079459608419154.
531 Bailey MT, Dowd SE, Parry NM, Galley JD, Schauer DB, Lyte M. 2010. Stressor exposure
532 disrupts commensal microbial populations in the intestines and leads to increased
533 colonization by *Citrobacter rodentium*. *Infection and immunity* 78:1509–1519. DOI:
534 10.1128/IAI.00862-09.
535 Bates D, Mächler M, Bolker B, Walker S. 2015. Fitting linear mixed-effects models using lme4.
536 *Journal of Statistical Software* 67:1–48. DOI: 10.18637/jss.v067.i01.
537 Bates KA, Shelton JMG, Mercier VL, Hopkins KP, Harrison XA, Petrovan SO, Fisher MC.
538 2019. Captivity and infection by the fungal pathogen *Batrachochytrium*
539 *salamandrivorans* perturb the amphibian skin microbiome. *Frontiers in Microbiology*
540 10:1834. DOI: 10.3389/fmicb.2019.01834.
541 Bébéar C, Pereyre S, Peuchant O. 2011. *Mycoplasma pneumoniae*: susceptibility and resistance
542 to antibiotics. *Future Microbiology* 6:423–431. DOI: 10.2217/fmb.11.18.
543 Becker MH, Richards-Zawacki CL, Gratwicke B, Belden LK. 2014. The effect of captivity on
544 the cutaneous bacterial community of the critically endangered Panamanian golden frog
545 (*Atelopus zeteki*). *Biological Conservation* 176:199–206. DOI:
546 10.1016/j.biocon.2014.05.029.

547 Becker MH, Walke JB, Murrill L, Woodhams DC, Reinert LK, Rollins-Smith LA, Burzynski
548 EA, Umile TP, Minbiole KP, Belden LK. 2015. Phylogenetic distribution of symbiotic
549 bacteria from Panamanian amphibians that inhibit growth of the lethal fungal pathogen
550 *Batrachochytrium dendrobatidis*. *Molecular Ecology* 24:1628–1641. DOI:
551 10.1111/mec.13135.
552 Benjamini Y, Hochberg Y. 1995. Controlling the false discovery rate: a practical and powerful
553 approach to multiple testing. *Journal of the Royal Statistical Society. Series B*
554 *(Methodological)* 57:289–300. DOI: 10.2307/2346101.
555 Bestion E, Jacob S, Zinger L, Di Gesu L, Richard M, White J, Cote J. 2017. Climate warming
556 reduces gut microbiota diversity in a vertebrate ectotherm. *Nature Ecology & Evolution*
557 1:1–3. DOI: 10.1038/s41559-017-0161.
558 Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, Alexander H, Alm
559 EJ, Arumugam M, Asnicar F, Bai Y, Bisanz JE, Bittinger K, Brejnrod A, Brislawn CJ,
560 Brown CT, Callahan BJ, Caraballo-Rodríguez AM, Chase J, Cope EK, Da Silva R,
561 Diener C, Dorrestein PC, Douglas GM, Durall DM, Duvallet C, Edwardson CF, Ernst M,
562 Estaki M, Fouquier J, Gauglitz JM, Gibbons SM, Gibson DL, Gonzalez A, Gorlick K,
563 Guo J, Hillmann B, Holmes S, Holste H, Huttenhower C, Huttley GA, Janssen S,
564 Jarmusch AK, Jiang L, Kaehler BD, Kang KB, Keefe CR, Keim P, Kelley ST, Knights
565 D, Koester I, Kosciolek T, Kreps J, Langille MGI, Lee J, Ley R, Liu Y-X, Loftfield E,
566 Lozupone C, Maher M, Marotz C, Martin BD, McDonald D, McIver LJ, Melnik AV,
567 Metcalf JL, Morgan SC, Morton JT, Naimey AT, Navas-Molina JA, Nothias LF,
568 Orchanian SB, Pearson T, Peoples SL, Petras D, Preuss ML, Pruesse E, Rasmussen LB,
569 Rivers A, Robeson MS, Rosenthal P, Segata N, Shaffer M, Shiffer A, Sinha R, Song SJ,

570 Spear JR, Swafford AD, Thompson LR, Torres PJ, Trinh P, Tripathi A, Turnbaugh PJ,
571 Ul-Hasan S, van der Hooft JJJ, Vargas F, Vázquez-Baeza Y, Vogtmann E, von Hippel M,
572 Walters W, Wan Y, Wang M, Warren J, Weber KC, Williamson CHD, Willis AD, Xu
573 ZZ, Zaneveld JR, Zhang Y, Zhu Q, Knight R, Caporaso JG. 2019. Reproducible,
574 interactive, scalable and extensible microbiome data science using QIIME 2. *Nature*
575 *Biotechnology* 37:852–857. DOI: 10.1038/s41587-019-0209-9.
576 Boon E, Meehan CJ, Whidden C, Wong DH-J, Langille MG, Beiko RG. 2014. Interactions in the
577 microbiome: communities of organisms and communities of genes. *FEMS Microbiology*
578 *Reviews* 38:90–118. DOI: 10.1111/1574-6976.12035.
579 Bornbusch SL, Harris RL, Grebe NM, Roche K, Dimac-Stohl K, Drea CM. 2021. Antibiotics
580 and fecal transfaunation differentially affect microbiota recovery, associations, and
581 antibiotic resistance in lemur guts. *Animal Microbiome* 3:65. DOI: 10.1186/s42523-021-
582 00126-z.
583 Broom LJ, Kogut MH. 2018. The role of the gut microbiome in shaping the immune system of
584 chickens. *Veterinary Immunology and Immunopathology* 204:44–51. DOI:
585 10.1016/j.vetimm.2018.10.002.
586 Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. 2016. DADA2:
587 High-resolution sample inference from Illumina amplicon data. *Nature Methods* 13:581–
588 583. DOI: 10.1038/nmeth.3869.
589 Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, Owens SM, Betley J,
590 Fraser L, Bauer M, Gormley N, Gilbert JA, Smith G, Knight R. 2012. Ultra-high-
591 throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms.
592 *The ISME Journal* 6:1621–1624. DOI: 10.1038/ismej.2012.8.

593 Ceruelos AH, Romero-Quezada LC, Ledezma JR, Contreras LL. 2019. Therapeutic uses of
594 metronidazole and its side effects: an update. *European Review for Medical and*
595 *Pharmacological Sciences* 23:397–401. DOI: 10.26355/eurrev_201901_16788.
596 Cheng Y, Fox S, Pemberton D, Hogg C, Papenfuss AT, Belov K. 2015. The Tasmanian devil
597 microbiome—implications for conservation and management. *Microbiome* 3:76. DOI:
598 10.1186/s40168-015-0143-0.
599 Clayton JB, Vangay P, Huang HU, Ward T, Hillmann BM, Al-Ghalith GA, Travis DA, Long
600 HT, Van Tuan B, Van Minh V. 2016. Captivity humanizes the primate microbiome.
601 *Proceedings of the National Academy of Sciences* 113:10376–10381. DOI:
602 10.1073/pnas.1521835113.
603 Connelly S, Subramanian P, Hasan NA, Colwell RR, Kaleko M. 2018. Distinct consequences of
604 amoxicillin and ertapenem exposure in the porcine gut microbiome. *Anaerobe* 53:82–93.
605 DOI: 10.1016/j.anaerobe.2018.04.012.
606 Cybulski W, Larsson P, Tjälve H, Kowalska-Pylka H, Sylla M, Semeniuk S. 1996. Disposition
607 of metronidazole in hens (*Gallus gallus*) and quails (*Coturnix coturnix japonica*):
608 pharmacokinetics and whole-body autoradiography. *Journal of Veterinary Pharmacology*
609 *and Therapeutics* 19:352–358. DOI: 10.1111/j.1365-2885.1996.tb00063.x.
610 Daskin JH, Alford RA. 2012. Context-dependent symbioses and their potential roles in wildlife
611 diseases. *Proceedings of the Royal Society B: Biological Sciences* 279:1457–1465. DOI:
612 10.1098/rspb.2011.2276.
613 Davidson GL, Somers SE, Wiley N, Johnson CN, Reichert MS, Ross RP, Stanton C, Quinn JL.
614 2021. A time-lagged association between the gut microbiome, nestling weight and

615 nestling survival in wild great tits. *Journal of Animal Ecology* 90:989–1003. DOI:
616 10.1111/1365-2656.13428.
617 Dorrestein GM. 2009. Passerines. *Handbook of Avian Medicine*:169–208. DOI: 10.1016/B978-0-
618 7020-2874-8.00008-0.
619 Escallón C, Becker MH, Walke JB, Jensen RV, Cormier G, Belden LK, Moore IT. 2017.
620 Testosterone levels are positively correlated with cloacal bacterial diversity and the
621 relative abundance of Chlamydiae in breeding male rufous-collared sparrows. *Functional*
622 *Ecology* 31:192–203. DOI: 10.1111/1365-2435.12696.
623 Faustino CR, Jennelle CS, Connolly V, Davis AK, Swarthout EC, Dhondt AA, Cooch EG. 2004.
624 *Mycoplasma gallisepticum* infection dynamics in a house finch population: seasonal
625 variation in survival, encounter and transmission rate. *Journal of Animal Ecology*
626 73:651–669. DOI: 10.1111/j.0021-8790.2004.00840.x.
627 Fernandes AD, Macklaim JM, Linn TG, Reid G, Gloor GB. 2013. ANOVA-like differential gene
628 expression analysis of single-organism and meta-RNA-seq. *PLoS one* 8:e67019.
629 Fernandes AD, Reid JN, Macklaim JM, McMurrough TA, Edgell DR, Gloor GB. 2014. Unifying
630 the analysis of high-throughput sequencing datasets: characterizing RNA-seq, 16S rRNA
631 gene sequencing and selective growth experiments by compositional data analysis.
632 *Microbiome* 2:1–13.
633 Fox J, Weisberg S. 2019. *An R Companion to Applied Regression, Third Edition*. Thousand Oaks
634 CA: Sage.
635 Grodio JL, Dhondt KV, O'Connell PH, Schat KA. 2008. Detection and quantification of
636 *Mycoplasma gallisepticum* genome load in conjunctival samples of experimentally

637 infected house finches (*Carpodacus mexicanus*) using real-time polymerase chain
638 reaction. *Avian Pathology* 37:385–391. DOI: 10.1080/03079450802216629.
639 Grond K, Sandercock BK, Jumpponen A, Zeglin LH. 2018. The avian gut microbiota:
640 community, physiology and function in wild birds. *Journal of Avian Biology* 49:e01788.
641 DOI: 10.1111/jav.01788.
642 Harris EV, Roode JC de, Gerardo NM. 2019. Diet–microbiome–disease: Investigating diet's
643 influence on infectious disease resistance through alteration of the gut microbiome. *PLoS*
644 *Pathogens* 15:e1007891. DOI: 10.1371/journal.ppat.1007891.
645 Hawley DM, Grodio J, Jr SF, Kirkpatrick L, Ley DH. 2011. Experimental infection of domestic
646 canaries (*Serinus canaria domestica*) with *Mycoplasma gallisepticum*: a new model
647 system for a wildlife disease. *Avian Pathology* 40:321–327. DOI:
648 10.1080/03079457.2011.571660.
649 Hernandez J, Escallón C, Medina D, Vernasco BJ, Walke JB, Belden LK, Moore IT. 2020.
650 Cloacal bacterial communities of tree swallows (*Tachycineta bicolor*): Similarity within a
651 population, but not between pair-bonded social partners. *PLoS ONE* 15:e0228982. DOI:
652 10.1371/journal.pone.0228982.
653 Hochachka WM, Dhondt AA. 2000. Density-dependent decline of host abundance resulting from
654 a new infectious disease. *Proceedings of the National Academy of Sciences of the United*
655 *States of America* 97:5303–5306. DOI: 10.1073/pnas.080551197.
656 Honda K, Littman DR. 2012. The microbiome in infectious disease and inflammation. *Annual*
657 *Review of Immunology* 30:759–795. DOI: 10.1146/annurev-immunol-020711-074937.
658 Hooper LV, Littman DR, Macpherson AJ. 2012. Interactions between the microbiota and the
659 immune system. *Science* 336:1268–1273. DOI: 10.1126/science.1223490.

660 Ichinohe T, Pang IK, Kumamoto Y, Peaper DR, Ho JH, Murray TS, Iwasaki A. 2011.
661 Microbiota regulates immune defense against respiratory tract influenza A virus
662 infection. *Proceedings of the National Academy of Sciences* 108:5354–5359. DOI:
663 10.1073/pnas.1019378108.
664 Kabat AM, Srinivasan N, Maloy KJ. 2014. Modulation of immune development and function by
665 intestinal microbiota. *Trends in Immunology* 35:507–517. DOI: 10.1016/j.it.2014.07.010.
666 Kalyana Chakravarthy S, Jayasudha R, Sai Prashanthi G, Ali MH, Sharma S, Tyagi M, Shivaji S.
667 2018. Dysbiosis in the gut bacterial microbiome of patients with uveitis, an inflammatory
668 disease of the eye. *Indian Journal of Microbiology* 58:457–469. DOI: 10.1007/s12088-
669 018-0746-9.
670 Kelly LW, Williams GJ, Barott KL, Carlson CA, Dinsdale EA, Edwards RA, Haas AF, Haynes
671 M, Lim YW, McDole T, Nelson CE, Sala E, Sandin SA, Smith JE, Vermeij MJA, Youle
672 M, Rohwer F. 2014. Local genomic adaptation of coral reef-associated microbiomes to
673 gradients of natural variability and anthropogenic stressors. *Proceedings of the National*
674 *Academy of Sciences* 111:10227–10232. DOI: 10.1073/pnas.1403319111.
675 Keohane DM, Woods T, O'Connor P, Underwood S, Cronin O, Whiston R, O'Sullivan O, Cotter
676 P, Shanahan F, Molloy MGM. 2019. Four men in a boat: Ultra-endurance exercise alters
677 the gut microbiome. *Journal of Science and Medicine in Sport* 22:1059–1064. DOI:
678 10.1016/j.jsams.2019.04.004.
679 Khosravi A, Mazmanian SK. 2013. Disruption of the gut microbiome as a risk factor for
680 microbial infections. *Current Opinion in Microbiology* 16:221–227. DOI:
681 10.1016/j.mib.2013.03.009.

682 Klomp JE, Murphy MT, Smith SB, McKay JE, Ferrera I, Reysenbach A-L. 2008. Cloacal
683 microbial communities of female spotted towhees *Pipilo maculatus*: microgeographic
684 variation and individual sources of variability. *Journal of Avian Biology* 39:530–538.
685 DOI: 10.1111/j.0908-8857.2008.04333.x.
686 Kogut MH, Lee A, Santin E. 2020. Microbiome and pathogen interaction with the immune
687 system. *Poultry science* 99:1906–1913. DOI: 10.1016/j.psj.2019.12.011.
688 Kohl K. 2012. Diversity and function of the avian gut microbiota. *Journal of Comparative*
689 *Physiology B: Biochemical, Systemic, and Environmental Physiology* 182:591–602. DOI:
690 10.1007/s00360-012-0645-z.
691 Kohl KD, Brun A, Bordenstein SR, Caviedes-Vidal E, Karasov WH. 2018. Gut microbes limit
692 growth in house sparrow nestlings (*Passer domesticus*) but not through limitations in
693 digestive capacity. *Integrative Zoology* 13:139–151. DOI: 10.1111/1749-4877.12289.
694 Kohl KD, Brun A, Magallanes M, Brinkerhoff J, Laspiur A, Acosta JC, Caviedes-Vidal E,
695 Bordenstein SR. 2017. Gut microbial ecology of lizards: insights into diversity in the
696 wild, effects of captivity, variation across gut regions and transmission. *Molecular*
697 *Ecology* 26:1175–1189. DOI: 10.1111/mec.13921.
698 Koskella B, Bergelson J. 2020. The study of host–microbiome (co)evolution across levels of
699 selection. *Philosophical Transactions of the Royal Society B* 375:20190604. DOI:
700 10.1098/rstb.2019.0604.
701 Kraemer SA, Ramachandran A, Perron GG. 2019. Antibiotic pollution in the environment: from
702 microbial ecology to public policy. *Microorganisms* 7:180. DOI:
703 10.3390/microorganisms7060180.

Lavrinienko A, Hämäläinen A, Hindström R, Tukalenko E, Boratyński Z, Kivisaari K, Mousseau TA, Watts PC, Mappes T. 2021. Comparable response of wild rodent gut microbiome to anthropogenic habitat contamination. *Molecular Ecology* 30:3485–3499. DOI: 10.1111/mec.15945.

Lazar V, Ditu L-M, Pircalabioru GG, Gheorghe I, Curutiu C, Holban AM, Picu A, Petcu L, Chifiriuc MC. 2018. Aspects of gut microbiota and immune system interactions in infectious diseases, immunopathology, and cancer. *Frontiers in Immunology* 9:1830. DOI: 10.3389/fimmu.2018.01830.

Leon AE, Hawley DM. 2017. Host responses to pathogen priming in a natural songbird host. *Ecohealth* 14:793–804. DOI: 10.1007/s10393-017-1261-x.

Ley DH, Hawley DM, Geary SJ, Dhondt AA. 2016. House finch (*Haemorhous mexicanus*) conjunctivitis, and *Mycoplasma* spp. isolated from North American wild birds, 1994–2015. *Journal of Wildlife Diseases* 52:669–673. DOI: 10.7589/2015-09-244.

Maurice CF, CL Knowles S, Ladau J, Pollard KS, Fenton A, Pedersen AB, Turnbaugh PJ. 2015. Marked seasonal variation in the wild mouse gut microbiota. *The ISME Journal* 9:2423–2434. DOI: 10.1038/ismej.2015.53.

McKnight DT, Huerlimann R, Bower DS, Schwarzkopf L, Alford RA, Zenger KR. 2019. Methods for normalizing microbiome data: An ecological perspective. *Methods in Ecology and Evolution* 10:389–400. DOI: 10.1111/2041-210X.13115.

Murray MH, Lankau EW, Kidd AD, Welch CN, Ellison T, Adams HC, Lipp EK, Hernandez SM. 2020. Gut microbiome shifts with urbanization and potentially facilitates a zoonotic pathogen in a wading bird. *PLoS ONE* 15:e0220926. DOI: 10.1371/journal.pone.0220926.

727 Nakamura YK, Metea C, Karstens L, Asquith M, Gruner H, Moscibrocki C, Lee I, Brislawn CJ,
728 Jansson JK, Rosenbaum JT, Lin P. 2016. Gut Microbial Alterations Associated With
729 Protection From Autoimmune Uveitis. *Investigative Ophthalmology & Visual Science*
730 57:3747–3758. DOI: 10.1167/iovs.16-19733.
731 Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, McGlinn D, Minchin PR, O'Hara
732 RB, Simpson GL, Solymos P, Stevens MHH, Szoecs E, Wagner H. 2018. vegan:
733 Community Ecology Package. R package version 2.5–2.
734 Oliveira BCM, Murray M, Tseng F, Widmer G. 2020. The fecal microbiota of wild and captive
735 raptors. *Animal Microbiome* 2:15. DOI: 10.1186/s42523-020-00035-7.
736 Oliver KM, Smith AH, Russell JA. 2014. Defensive symbiosis in the real world–advancing
737 ecological studies of heritable, protective bacteria in aphids and beyond. *Functional*
738 *Ecology* 28:341–355. DOI: 10.1111/1365-2435.12133.
739 Pallav K, Dowd SE, Villafuerte J, Yang X, Kabbani T, Hansen J, Dennis M, Leffler DA,
740 Newburg DS, Kelly CP. 2014. Effects of polysaccharopeptide from *Trametes versicolor*
741 and amoxicillin on the gut microbiome of healthy volunteers: a randomized clinical trial.
742 *Gut Microbes* 5:458–467. DOI: 10.4161/gmic.29558.
743 Paradis E, Schliep K. 2019. ape 5.0: an environment for modern phylogenetics and evolutionary
744 analysis in R. *Bioinformatics* 35:526–528.
745 Pélissier M-A, Vasquez N, Balamurugan R, Pereira E, Dossou-Yovo F, Suau A, Pochart P,
746 Magne F. 2010. Metronidazole effects on microbiota and mucus layer thickness in the rat
747 gut. *FEMS Microbiology Ecology* 73:601–610. DOI: 10.1111/j.1574-6941.2010.00916.x.
748 R Development Core Team. 2015.R: a language and environment for statistical computing.
749 *Available at http://www.gbif.org/resource/81287*

Rosshart SP, Vassallo BG, Angeletti D, Hutchinson DS, Morgan AP, Takeda K, Hickman HD, McCulloch JA, Badger JH, Ajami NJ. 2017. Wild mouse gut microbiota promotes host fitness and improves disease resistance. *Cell* 171:1015–1028. DOI: 10.1016/j.cell.2017.09.016.

RStudio Team. 2020. *RStudio: Integrated Development Environment for R. RStudio, PBC., Boston, MA, USA*.

Salgado-Flores A, Tveit AT, Wright A-D, Pope PB, Sundset MA. 2019. Characterization of the cecum microbiome from wild and captive rock ptarmigans indigenous to Arctic Norway. *PLoS ONE* 14:e0213503. DOI: 10.1371/journal.pone.0213503.

San Juan PA, Castro I, Dhami MK. 2021. Captivity reduces diversity and shifts composition of the Brown Kiwi microbiome. *Animal Microbiome* 3:48. DOI: 10.1186/s42523-021-00109-0.

Sarkar A, Harty S, Johnson KV-A, Moeller AH, Archie EA, Schell LD, Carmody RN, Clutton-Brock TH, Dunbar RI, Burnet PW. 2020. Microbial transmission in animal social networks and the social microbiome. *Nature Ecology & Evolution* 4:1020–1035. DOI: 10.1038/s41559-020-1220-8.

Schokker D, Jansman AJ, Veninga G, de Bruin N, Vastenhouw SA, de Bree FM, Bossers A, Rebel JM, Smits MA. 2017. Perturbation of microbiota in one-day old broiler chickens with antibiotic for 24 hours negatively affects intestinal immune development. *BMC Genomics* 18:1–14. DOI: 10.1186/s12864-017-3625-6.

Schuijt TJ, Lankelma JM, Scicluna BP, e Melo F de S, Roelofs JJ, de Boer JD, Hoogendijk AJ, de Beer R, de Vos A, Belzer C. 2016. The gut microbiota plays a protective role in the

772 host defence against pneumococcal pneumonia. *Gut* 65:575–583. DOI: 10.1136/gutjnl-
773 2015-309728.
774 Shukla SD, Budden KF, Neal R, Hansbro PM. 2017. Microbiome effects on immunity, health
775 and disease in the lung. *Clinical & Translational Immunology* 6:e133. DOI:
776 10.1038/cti.2017.6.
777 Song SJ, Sanders JG, Delsuc F, Metcalf J, Amato K, Taylor MW, Mazel F, Lutz HL, Winker K,
778 Graves GR, Humphrey G, Gilbert JA, Hackett SJ, White KP, Skeen HR, Kurtis SM,
779 Withrow J, Braile T, Miller M, McCracken KG, Maley JM, Ezenwa VO, Williams A,
780 Blanton JM, McKenzie VJ, Knight R. 2020. Comparative analyses of vertebrate gut
781 microbiomes reveal convergence between birds and bats. *mBio* 11:e02901-19. DOI:
782 10.1128/mBio.02901-19.
783 Sydenstricker KV, Dhondt AA, Ley DH, Kollias GV. 2005. Re-exposure of captive house
784 finches that recovered from *Mycoplasma gallisepticum* infection. *Journal of Wildlife*
785 *Diseases* 41:326–333. DOI: 10.7589/0090-3558-41.2.326.
786 Teyssier A, Rouffaer LO, Hudin NS, Strubbe D, Matthysen E, Lens L, White J. 2018. Inside the
787 guts of the city: urban-induced alterations of the gut microbiota in a wild passerine.
788 *Science of the Total Environment* 612:1276–1286. DOI: 10.1016/j.scitotenv.2017.09.035.
789 Thomason CA, Leon A, Kirkpatrick LT, Belden LK, Hawley DM. 2017. Eye of the Finch:
790 characterization of the ocular microbiome of house finches in relation to mycoplasmal
791 conjunctivitis. *Environmental Microbiology* 19:1439–1449. DOI: 10.1111/1462-
792 2920.13625.
793 Trevelline BK, Fontaine SS, Hartup BK, Kohl KD. 2019. Conservation biology needs a
794 microbial renaissance: a call for the consideration of host-associated microbiota in

795 wildlife management practices. *Proceedings of the Royal Society B: Biological Sciences*
796 286:20182448. DOI: 10.1098/rspb.2018.2448.
797 Videvall E, Strandh M, Engelbrecht A, Cloete S, Cornwallis CK. 2018. Measuring the gut
798 microbiome in birds: comparison of faecal and cloacal sampling. *Molecular Ecology*
799 *Resources* 18:424–434. DOI: 10.1111/1755-0998.12744.
800 Vieira WA, Pretorius E. 2010. The impact of asthma on the gastrointestinal tract (GIT). *Journal*
801 *of Asthma and Allergy* 3:123. DOI: 10.2147/JAA.S10592.
802 Vinkler M, Leon AE, Kirkpatrick L, Dalloul RA, Hawley DM. 2018. Differing house finch
803 cytokine expression responses to original and evolved isolates of *Mycoplasma*
804 *gallisepticum*. *Frontiers in Immunology* 9:13. DOI: 10.3389/fimmu.2018.00013.
805 Vlčková K, Shutt-Phillips K, Heistermann M, Pafčo B, Petrželková KJ, Todd A, Modrý D,
806 Nelson KE, Wilson BA, Stumpf RM, White BA, Leigh SR, Gomez A. 2018. Impact of
807 stress on the gut microbiome of free-ranging western lowland gorillas. *Microbiology*
808 164:40–44. DOI: 10.1099/mic.0.000587.
809 Walke JB, Belden LK. 2016. Harnessing the microbiome to prevent fungal infections: lessons
810 from amphibians. *PLoS Pathogens* 12:e1005796. DOI: 10.1371/journal.ppat.1005796.
811 Wang J, Li F, Wei H, Lian Z-X, Sun R, Tian Z. 2014. Respiratory influenza virus infection
812 induces intestinal immune injury via microbiota-mediated Th17 cell–dependent
813 inflammation. *Journal of Experimental Medicine* 211:2397–2410. DOI:
814 10.1084/jem.20140625.
815 Weitzman CL, Rostama B, Thomason CA, May M, Belden LK, Hawley DM. 2021.
816 Experimental test of microbiome protection across pathogen doses reveals importance of

817 resident microbiome composition. *FEMS Microbiology Ecology* 97:fiab141. DOI:
818 10.1093/femsec/fiab141.
819 Weyrich LS, Feaga HA, Park J, Muse SJ, Safi CY, Rolin OY, Young SE, Harvill ET. 2014.
820 Resident microbiota affect *Bordetella pertussis* infectious dose and host specificity. *The*
821 *Journal of Infectious Diseases* 209:913–921. DOI: 10.1093/infdis/jit597.
822 Wilks J, Golovkina T. 2012. Influence of microbiota on viral infections. *PLoS Pathogens*
823 8:e1002681. DOI: 10.1371/journal.ppat.1002681.
824 Wise MG, Siragusa GR. 2007. Quantitative analysis of the intestinal bacterial community in one-
825 to three-week-old commercially reared broiler chickens fed conventional or antibiotic-
826 free vegetable-based diets. *Journal of Applied Microbiology* 102:1138–1149. DOI:
827 10.1111/j.1365-2672.2006.03153.x.
828 Worsley SF, Davies CS, Mannarelli M-E, Hutchings MI, Komdeur J, Burke T, Dugdale HL,
829 Richardson DS. 2021. Gut microbiome composition, not alpha diversity, is associated
830 with survival in a natural vertebrate population.
831 Xiong W, Wang Y, Sun Y, Ma L, Zeng Q, Jiang X, Li A, Zeng Z, Zhang T. 2018. Antibiotic-
832 mediated changes in the fecal microbiome of broiler chickens define the incidence of
833 antibiotic resistance genes. *Microbiome* 6:1–11. DOI: 10.1186/s40168-018-0419-2.
834 Yuan ML, Dean SH, Longo AV, Rothermel BB, Tuberville TD, Zamudio KR. 2015. Kinship,
835 inbreeding and fine-scale spatial structure influence gut microbiota in a hindgut-
836 fermenting tortoise. *Molecular Ecology* 24:2521–2536. DOI: 10.1111/mec.13169.
837
838
839
840
841
842

Table 1. Experimental design. House finches were randomly assigned to receive oral antibiotics in their water and inoculated with *Mycoplasma gallisepticum* (MG) or sham control. Values in parentheses represent sample sizes for 16S rRNA gene amplicon sequencing of cloacal swabs. These cloacal swab samples were collected on day –1 (after oral antibiotics but prior to MG inoculation), with MG sham control samples from day 7 also sequenced to detect the extended effects of oral antibiotics.

Sample Sizes:	**MG control (sham)**	**MG**
Microbiome control (no antibiotics)	n = 8 (8)	n =17 (12)
Antibiotics	n = 8 (8)	n = 17 (5)

Figure 1. Experimental timeline. Wild-caught house finches were administered oral antibiotics for one week prior to inoculation with *Mycoplasma gallisepticum*. Throughout the course of disease, we monitored pathology and pathogen load by scoring eye lesions and collecting eye swabs (E) and detected differences in antibody responses with blood samples (B). We also collected cloacal swabs to detect short- and long-term effects of oral antibiotics on cloacal bacterial communities (C). A subset of cloacal swabs were 16S rRNA gene amplicon sequenced (see *Methods*).

(new fig: Fig1_Timeline_20211217.pdf)

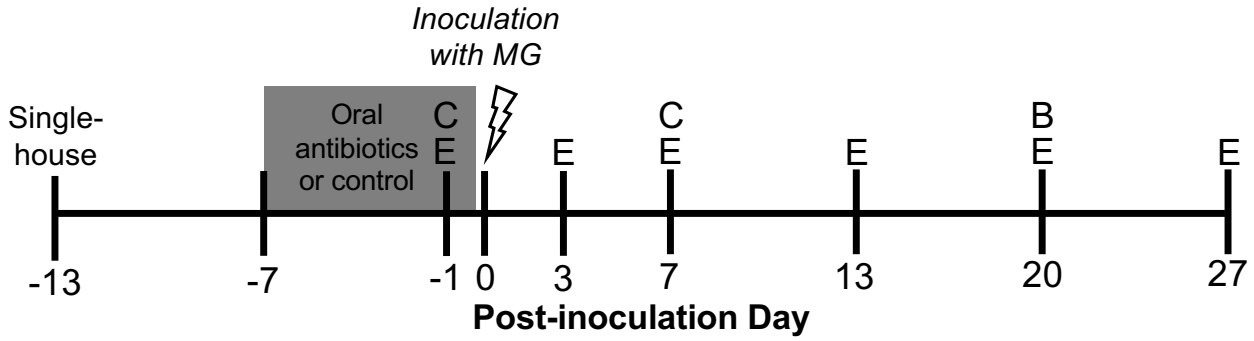


Figure 2. Mycoplasmal disease in house finches was not significantly affected by pre-inoculation oral antibiotics. (A) Pathology eye scores (left plus right eye scores) and (B) MG loads (log_{10}(load+1)) in the conjuctiva by experimental treatment group (n = 8–10 per point) across the course of disease. MG loads for sham-inoculated birds were only measured on day 3 post-inoculation. Values are averages ± standard error of raw data.

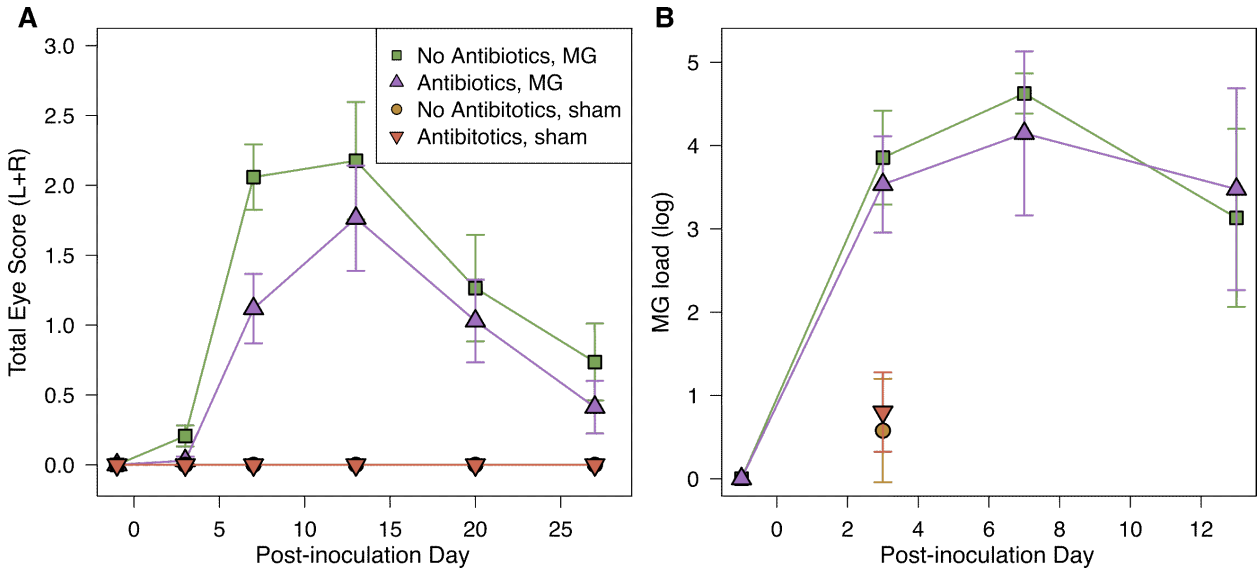
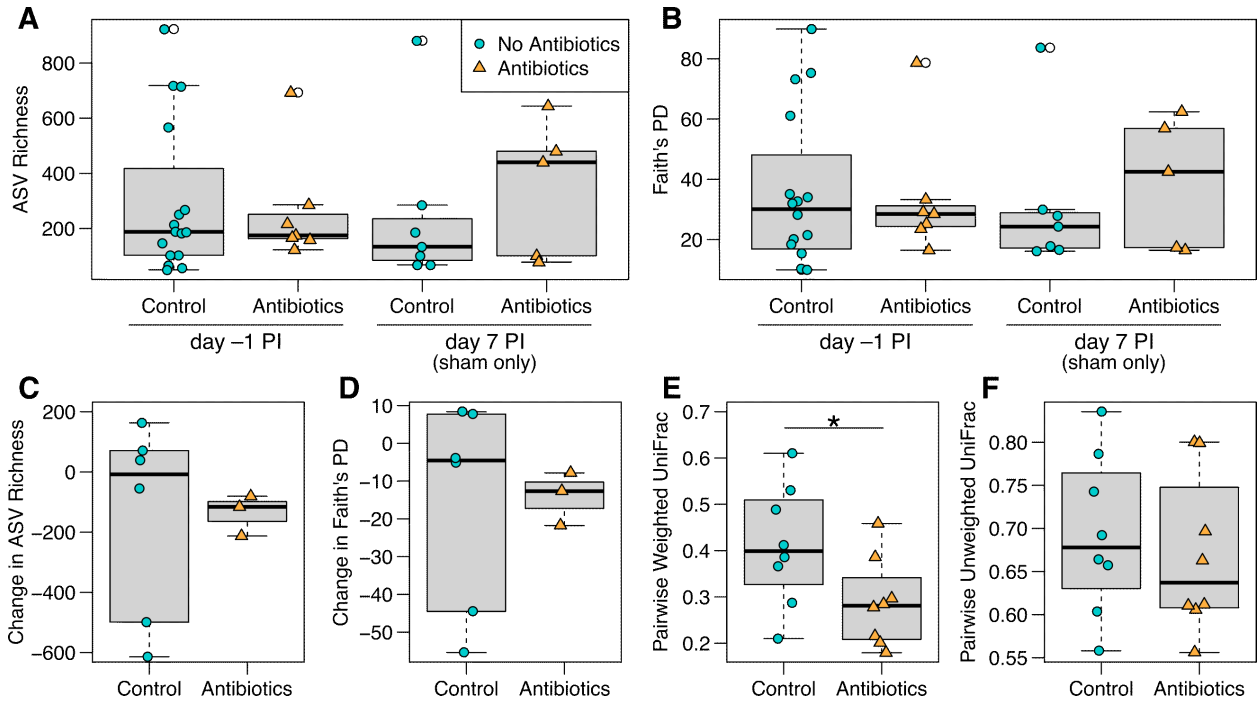


Figure 3. ASV richness and phylogenetic diversity in captive house finch cloacal swab samples. (A) Richness and (B) Faith's phylogenetic diversity by antibiotics treatment group and day sampled, with day –1 post-inoculation (PI) representing the sixth day of antibiotics or control treatment. (C,D) Change in richness and phylogenetic diversity from day –1 to day 7 post-inoculation within each individual bird. (E,F) Pairwise weighted and unweighted UniFrac distances within individuals from day –1 to 7, where greater values indicate less similarity. Day 7 values represent MG-sham birds only.

878


A
No Antibiotics
Antibiotics
ASV Richness
800
600
400
200
Control
Antibiotics
Control
Antibiotics
day −1 PI
day 7 PI
(sham only)
B
Faith's PD
80
60
40
20
Control
Antibiotics
Control
Antibiotics
day −1 PI
day 7 PI
(sham only)
C
Change in ASV Richness
200
0
−200
−400
−600
Control
Antibiotics
D
Change in Faith's PD
10
0
−10
−20
−30
−40
−50
Control
Antibiotics
E
Pairwise Weighted UniFrac
0.7
0.6
0.5
0.4
0.3
0.2
*
Control
Antibiotics
F
Pairwise Unweighted UniFrac
0.80
0.75
0.70
0.65
0.60
0.55
Control
Antibiotics

879
880