House Finches with High Coccidia Burdens Experience More Severe Experimental *Mycoplasma gallisepticum* Infections

Chava L. Weitzman[1*] ORCID 0000-0002-6103-1885

Courtney Thomason[1,2*] ORCID 0000-0003-2316-3518

Edward J. A. Schuler[1,3*] ORCID 0000-0002-1847-5712

Ariel Leon[1] ORCID 0000-0001-9246-4619

Sara R. Teemer[1]

Dana M. Hawley[1] ORCID 0000-0001-9573-2914

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

[2] Tennessee Department of Environment and Conservation, Division of Remediation, Oak Ridge, TN

[3] Department of Microbiology and Immunology, Virginia Commonwealth Medical Center, Richmond, VA

* authors contributed equally

Correspondence to: Chava Weitzman, clweitzman@vt.edu

Abstract

Parasites co-infecting hosts can interact directly and indirectly to affect parasite growth and disease manifestation. We examined potential interactions between two common parasites of house finches: the bacterium *Mycoplasma gallisepticum* that causes conjunctivitis and the intestinal coccidian parasite *Isospora* sp. We quantified coccidia burdens prior to and following experimental infection with *M. gallisepticum*, exploiting the birds' range of natural coccidia

burdens. Birds with greater baseline coccidia burdens developed higher *M. gallisepticum* loads and longer lasting conjunctivitis following inoculation. However, experimental inoculation with *M. gallisepticum* did not appear to alter coccidia shedding. Our study suggests that differences in immunocompetence or condition may predispose some finches to more severe infections with both pathogens.

Keywords:

co-infection, coccidia, conjunctivitis, *Mycoplasma gallisepticum*

Acknowledgements: Thank you to two anonymous reviewers for their comments on this manuscript.

Declarations:

Funding: This work was funded by NIH grant 5R01GM105245 as part of the joint NIH-NSF-USDA Ecology and Evolution of Infectious Diseases program. Funding for E. Schuler was provided by a Virginia Tech Summer Undergraduate Research Fellowship. C. Weitzman was supported by National Science Foundation grant IOS-1755051.

Conflicts of interest: On behalf of all authors, the corresponding author states that there is no conflict of interest.

Ethics Approval: Birds were captured under VDGIF (050352) and USFWS (MB158404-1) permits. Experimental procedures were approved by Virginia Tech's Institutional Animal Care and Use Committee.

Consent to participate: NA

Consent for publication: NA

Data availability: The dataset analyzed during this study are available from the corresponding author on reasonable request.

Code availability: NA

Introduction

Free-living hosts typically house a complex suite of parasites and pathogens that interact with each other, and their host, through bottom-up (resource-mediated) and top-down (immune-mediated) processes (Pedersen and Fenton 2007). Indirect interactions such as immune-mediated processes, whereby parasites interact with each other through modulation of their host, are particularly relevant for co-infecting parasites that do not occupy the same host tissues. For example, in humans, immune stimulation by the gastrointestinal pathogen *Helicobacter pylori* suppresses co-infection of the lung pathogen *Mycobacterium tuberculosis* (TB) as a consequence of both infections stimulating T-helper 1 (Th1) immunity (Perry et al. 2010). When two parasites stimulate different immune components, an immunological bias against one invader can facilitate a second parasite's invasion or severity. In buffalo, individuals that produced a strong T-helper type 2 (Th2) response to combat nematode infections had lower Th1 immunity and were more likely to be invaded by bovine TB (Ezenwa et al. 2010). Regardless of the specific mechanisms by which co-infecting parasites interact, these interactions often impact the outcome of disease and ultimately affect host fitness.

Here, we examine potential immune-mediated interactions between a conjunctival pathogen (*Mycoplasma gallisepticum*, hereafter "MG") and gut parasite (coccidian protozoa), which both naturally occur in house finches (*Haemorhous mexicanus*). MG first appeared in house finches in the mid-1990s and since spread to much of the species' distribution (Ley et al.

2016). This infection causes severe inflammation in the conjunctivae and high host mortality in the wild (Faustino et al. 2004). Coccidia, an umbrella term for various species of intestinal protozoan parasites, naturally infect the finch gut. Coccidia infections of *Isospora* spp. (the culprit in house finches; Brawner III et al. 2000; Hartup et al. 2004) damage intestinal epithelial walls, decreasing absorption of nutrients and body mass (Hõrak et al. 2004). Both MG and coccidia commonly cause disease in wild house finches (Giraudeau et al. 2014; Ley et al. 2016) with severe fitness consequences, and they co-occur in nature (Brawner III et al. 2000; Hartup et al. 2004). Therefore, we examined whether MG and coccidia interact with each other in a way that may alter the outcome of either infection.

We tested for interactions between these parasites in house finches by quantifying oocyst shedding from natural coccidia infections prior to and following experimental inoculation with MG. Our goal was to evaluate the degree and nature (synergistic or antagonistic) of potential interactions between coccidia (*Isospora* sp.) and MG. We predicted an antagonistic interaction due to previous studies that have identified similar T-helper subset (i.e., Th1) responses to both pathogens (Yun et al. 2000; Vinkler et al. 2018). Co-infection would likely stimulate strong Th1 responses and lead to the decline of one or both pathogens via immune-mediated interactions.

Methods

Hatch-year house finches were captured June–August 2015 in Blacksburg and Radford, Virginia. Only birds without conjunctival pathology throughout a quarantine period, seronegative for MG (Hawley et al. 2011), and negative for MG via quantitative PCR (Grodio et al. 2008) were included. Finches were single-housed two weeks before MG inoculation, with *ad libitum* food and water and a 12:12 light:dark photoperiod. To minimize mortality due to coccidiosis,

which can be high in captivity, finches were given sulfadimethoxine (5 days at 0.469 mg/ml followed by daily 0.26 mg/mL) in their water, but treatment ceased 14 days prior to experimental inoculation with MG. Treatment temporarily lowers coccidia loads, but does not clear infection (Brawner III et al. 2000); thus, many birds harbored high loads pre-inoculation (see *Results*).

To examine potential interactions between MG and coccidia, we sampled coccidia oocyst burdens from 42 total finches (20 MG-infected, 22 sham-inoculated controls) both prior to and following experimental MG inoculation. These 42 birds were a subset of a larger MG inoculation study (Leon et al. 2019), and treatment groups were assigned naïve to coccidia status.

On inoculation day (day 0), finches in the MG-infected treatment (10 female, 10 male) were inoculated in both eyes with 70 µL of the VA-1994 (7994-1 7 P 2/12/09) MG isolate in Frey's broth media (1 x 10^6 color changing units/mL concentration); control finches (12 female, 10 male) were sham inoculated with sterile Frey's media. We scored pathology six times from day 3–34 post-inoculation (Fig. 1b) on a 0–3 scale per eye and summed values between the two eyes for a maximum value of 6 (Sydenstricker et al. 2006). Conjunctival swabs on post-inoculation days 6 and 20 were used to quantify MG load via quantitative PCR as per Leon et al. 2019.

Fecal samples were collected 7, 4, and 3 days prior to inoculation to determine baseline coccidia loads, and on days 2 and 5 post-inoculation to detect changes early in MG infection. As shedding of coccidia oocysts varies temporally, peaking in late afternoon (Brawner III and Hill 1999), fresh fecal samples were collected from 17:00–17:30. Fecal samples were stored in 1mL of 10% formalin at 4˚C, and oocysts were counted using standard fecal float analysis with a Fecalyzer (EVSCO Pharmaceuticals) and Sheather's sugar solution. To adhere floating oocysts, a glass coverslip was placed over the reverse meniscus for 15 minutes, and total oocysts were

117 counted via bright-field microscopy at 100x magnification. Results are presented in units of
118 oocysts per gram of feces (OPG).
119 All statistical analyses were conducted using R ver 3.5.3 in R Studio ver 1.1.463 (R
120 Development Core Team 2015; RStudio Team 2016). Significance was determined with Type II
121 or III Wald tests, where appropriate, with the car package (Fox and Weisberg 2019). Models
122 were simplified using sequential deletion of covariates (i.e., sex, post-inoculation day) with p-
123 values > 0.1 either as main effects or in interaction with other fixed effects. Below, we note cases
124 where variables were removed from our final models.
125 To determine how infection with MG affects coccidia, we asked how coccidia loads
126 changed over time with experimental treatment (infected versus control). We excluded
127 individuals that never shed oocysts on any sampling day from this analysis (n = 5) since we were
128 specifically interested in how MG treatment altered existing coccidia infections. We used the
129 glmmTMB package (Brooks et al. 2017) to run zero-inflated negative binomial generalized
130 linear mixed effects models, asking whether coccidia loads differed before and after MG
131 inoculation for experimentally-infected versus control birds (MG treatment*pre/post
132 inoculation). Sex was included as a covariate. Because birds were housed among four rooms
133 (evenly representing the treatments), and room was a significant predictor of coccidia load, we
134 included bird ID nested within room as a random variable.
135 To determine how infection with coccidia affects responses to MG, we analyzed whether
136 naturally-occurring variation in coccidia shedding predicted MG load and pathology using birds
137 from the MG-infected treatment alone. We calculated average pre-inoculation coccidia loads
138 ($\log_{10}$(load+1)) as a baseline and used that to predict resulting MG responses. To determine if
139 baseline coccidia loads predicted the course of mycoplasmal infections, we modeled MG loads

($\log_{10}$(load + 1)) as a function of baseline coccidia loads and time (pre-inoculation coccidia*post-inoculation day) using linear mixed effects models in the lme4 package (Bates et al. 2015). We treated post-inoculation day as ordinal because MG loads were measured twice. Sex was included as a covariate and bird ID as a random variable. Room was not included here, because unlike coccidia, MG does not spread among separately housed birds within a room (this was verified using MG load residuals, which centered on zero across housing rooms). Pathology scores were similarly modeled using ordinal logistic regression with a cumulative link mixed model in the package ordinal (Christensen 2019). Here, post-inoculation day was a continuous variable.

Results and Discussion

Coccidia was common among our study birds, with 40–69% of the birds shedding oocysts on any sampling day (at burdens ranging from 18–60,400 OPG), and only five birds never shedding oocysts. Interestingly, the sexes differed in coccidia loads ($X^2 = 6.26$, df = 1, p = 0.01), with higher and more variable oocyst loads in male versus female finches (Male 95% CI = 575–3143 OPG; Female 95% CI = 129–690 OPG). Thus, sex was retained in our model of coccidia burdens.

We expected average coccidia burdens to decline after MG inoculation, resulting in a significant interaction between MG treatment and pre/post inoculation, but we found no such effect on coccidia oocyst count ($X^2 = 0.31$, df = 1, p > 0.5). It is possible that our sampling, which only extended to day 5 post-MG inoculation, ended too early to detect a change in coccidia shedding due to MG. A recent study found that several pro-inflammatory cytokines peak in finch harderian glands at day 6 post-MG inoculation (Vinkler et al. 2018), though there

are no comparable data for intestinal tissue to assess the timing of cytokine expression. In another co-infection study on house finches, *Plasmodium* infection intensity increased after MG inoculation, with the strongest effects occurring in the second week of MG infection (Reinoso-Pérez et al. 2020). Thus, sampling coccidia burdens for longer after MG inoculation may allow time for birds to fully develop the immune responses to MG that we expected to potentially affect co-infecting coccidia.

Conversely, we predicted that birds with high coccidia burdens prior to inoculation would develop relatively lower MG loads and conjunctivitis pathology due to high baseline immune stimulation. Instead, we found greater MG loads and disease in birds shedding more coccidia oocysts prior to inoculation (Fig. 1). Finches with higher baseline coccidia burdens developed higher MG loads ($X^2 = 4.47$, df = 1, $p = 0.03$; Fig. 1a), and MG loads significantly varied between post-inoculation days ($X^2 = 196$, df = 1, $p < 0.0001$). Sex and the interaction between coccidia burden and post-inoculation day were not retained in the final MG load model ($p > 0.1$).

For pathology, finches with higher baseline coccidia burdens showed lower and later average peaks in conjunctival pathology, but harbored clinical pathology for longer than birds with lower baseline coccidia burdens (coccidia*post-inoculation day $z = 2.67$, $p = 0.008$; Fig. 1b). Though the interaction between baseline coccidia burden and post-inoculation day was significant, baseline coccidia alone did not predict MG pathology ($z = –0.70$, $p = 0.5$). However, pathology score was significantly predicted by host sex ($z = –2.74$, $p = 0.006$) and post-inoculation day ($z = –3.57$, $p = 0.0004$), and thus both were retained in the final model.

In another co-infection study in house finches, Dhondt and colleagues (2017) found that finches with chronic baseline *Plasmodium* infections had greater MG load and disease severity when experimentally infected with MG. Because both Dhondt et al. (2017) and our study here

relied on naturally-occurring variation in a chronic infection (coccidia or avian malaria) prior to experimental infection with MG, it is impossible to determine whether the more severe infection and prolonged disease observed in birds with higher initial coccidia burdens reflects a true causal interaction. It is possible that birds with higher baseline coccidia loads did, in fact, harbor higher chronic immune stimulation prior to MG inoculation. However, rather than suppressing MG responses once inoculated, Th1-mediated responses could instead have lengthened the period of clinical conjunctivitis, as a previous study found associations between Th1 responses and conjunctivitis severity in this system (Vinkler et al. 2018). More likely, our correlational results reflect some underlying trait of immune competence influencing responses to both infections. For example, finches with lower coccidia burdens may have stronger immune systems capable of keeping diverse types of parasites, including MG, at bay. Importantly, co-infection between MG and coccidia could affect other variables not measured here, including susceptibility to other parasites or pathogens.

MG became an emerging infectious disease in house finches in the mid-1990s (Ley et al. 1996), invading hosts already parasitized by many other species, including coccidia. Our results, alongside those of Dhondt and colleagues (2017), suggest that the presence of other parasites is an important predictive factor for understanding the severity of MG infection and clinical disease. These co-infections, including others not addressed here, are thus an important consideration in understanding effects of MG on house finch fitness and transmission potential.

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Figure Caption

Fig. 1 Coccidia shedding prior to *Mycoplasma gallisepticum* (MG) inoculation predicts (a) MG load and (b) severity of pathology to MG over the course of disease. Although coccidia shedding was analyzed as a continuous variable, data are grouped here for visualization purposes. Filled circles = no coccidia prior to MG inoculation, open triangles = low coccidia shedding (average < 250 oocysts per gram), filled triangles = high coccidia shedding (average 1700–8500 oocysts per gram)