Modulating disease phenotype in a songbird: A role for inflammation in disease tolerance?

Running title: Modulating disease phenotype in a songbird

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Abstract

Individual animals vary greatly in their responses to infection, either killing off the invading pathogen (resistance) or minimizing the per-pathogen costs of infection on host fitness (tolerance). Though we understand little about the physiological drivers of tolerance in wild animals, phenotypically, it manifests as milder clinical signs of disease. Here, we use a well described disease system, finch mycoplasmosis, to evaluate the role of inflammation in disease tolerance. House finches (*Haemorhous mexicanus*) infected with the bacterial pathogen *Mycoplasma gallisepticum* (MG) develop conjunctival pathology that satisfies the cardinal signs of inflammation. We report on a captive trial performed in 2016 and replicated in 2018 that tested whether chemotherapeutics, specifically non-steroidal anti-inflammatory drugs (NSAIDs), can reduce lesion severity, thus pushing individuals toward more tolerant phenotypes. Though birds treated with NSAIDs in the first trial developed milder pathology per unit pathogen load, we found no effect of treatment in the second trial, perhaps due to natural variation in baseline tolerance within the source population across years. Second-trial control birds developed markedly milder pathology than first-year controls, suggesting that the effect of trial swamped the effect of treatment in this study. Moving forward, using birds from a population in which the disease is absent or only recently emerged—and so tolerance has not yet been selected for—may better elucidate the role of pro-inflammatory mediators in disease tolerance.

Keywords: disease tolerance, inflammation, non-steroidal anti-inflammatory drugs (NSAIDs), phenotype

Research Highlights: An individual's disease phenotype reflects how its immune system responds to tissue injury caused by an infectious pathogen. Limiting the pro-inflammatory response may be one way animals express milder clinical signs to disease consistent with disease tolerance. Exogenous treatment with non-steroidal anti-inflammatory drugs may similarly push individuals toward tolerant phenotypes, however, population-level swings in baseline tolerance can mask treatment effects.

Introduction

When the immune system detects infection, it can kill the invading pathogen (resistance) or minimize per-pathogen reductions to host fitness (tolerance) (Råberg, 2014). Though resistance permits pathogen clearance with the potential for sterilizing immunity, it also poses a risk for collateral tissue damage ('immunopathology') and exacerbated sickness behaviors that can reduce fitness in the immediate term (Adelman and Hawley, 2017). In contrast, tolerance permits pathogen persistence by minimizing the costs to the host of pathogen invasion and replication. Rather, animals can harbor high pathogen loads while maintaining tissue function and expressing normal, fitness-enhancing behaviors, which may increase the probability of contacting conspecifics and spreading pathogens (Adelman and Hawley, 2017; Burgan, Gervasi, Johnson and Martin, 2019; Martin *et al.*, 2019). Thus, while tolerance may be an adaptive survival strategy for individual hosts, it could also support pathogen fitness via increased transmission to new hosts **(**Råberg, Graham and Read, 2009; Boots, Best, Miller and White, 2009; Sears, Rohr, Allen and Martin, 2011). Identifying the mechanisms that mediate host tolerance at the individual-level can therefore improve our understanding of infectious disease dynamics in the wild.

Despite the potential for strong effects on epidemic outcomes, the physiological mechanisms underlying tolerance in animals remain largely unknown (Råberg, 2014). Among vertebrates, one promising mechanism involves dampening the pro-inflammatory response to infection, responsible for local swelling, release of pro-inflammatory cytokines, and production of free radicals (Graham, Allen and Read, 2005; Råberg *et al.*, 2009; Sears *et al*., 2011). Because these responses can induce significant damage to a host's own tissues, incurring serious fitness costs, reducing such inflammatory mediators could preserve fitness during infection and increase tolerance ('damage limitation,' see Vale, Fenton and Brown, 2014 for review). Of note, tolerance

and pathogen clearance need not be mutually exclusive (Restif and Koella, 2003; Restif and Koella, 2004). For instance, a host could still mount a targeted, adaptive immune response in the absence of over-exuberant inflammation, effectively reducing pathogen burden while also minimizing self-harm (Hurtado, 2012; Adelman, Kirkpatrick, Grodio and Hawley, 2013; Bonneaud *et al.*, 2019). In the experiments reported here, we explore the potential for reduced inflammation to enhance tolerance, while still allowing the expression of effective resistance mechanisms. To do so, we test the effect of non-steroidal anti-inflammatory drugs (NSAIDs) on host responses in an ecologically relevant host-pathogen system, house finches (*Haemorhous mexicanus*) experimentally infected with *Mycoplasma gallisepticum* (MG).

MG is a bacterial pathogen of poultry that was first isolated in North American songbirds in the mid-1990s (Ley, Berkhoff and McLaren, 1996; Delaney *et al.*, 2012). House finches are highly susceptible to infection, and the initial outbreak decimated populations in the eastern United States (Hochachka and Dhondt, 2000). In subsequent decades, MG has become endemic in house finch populations across North America and seasonal epidemics continue annually (Hosseini, Dhondt and Dobson, 2004; Dhondt *et al.*, 2005).

Finch isolates of MG have a tropism for the soft tissues surrounding the eye or conjunctiva (Ley *et al.*, 1996). In addition to inducing sickness behaviors like lethargy and anorexia, infection with MG can cause gross pathology that satisfies the cardinal signs of inflammation, including: periocular swelling (*tumor*), increased perfusion leading to erythema (*rubor*) and heat (*calor*), blepharospasm, or squinting, which can be used as a proxy for pain (*dolor*), and impaired vision (*functio laesa*) (Rather, 1971). Infection can also cause tissues to become leaky, or exudative, such that purulent discharge functionally seals the eye (Ley *et al.*, 1996). Given the importance of inflammation to disease progression (Luttrell, Fischer,

Stallknecht, and Kleven, 1996; Hurtado, 2012; Adelman *et al.*, 2013), finch mycoplasmosis provides an ideal system for exploring its role in tolerant versus resistant disease phenotypes.

Here, we test whether a nonsteroidal anti-inflammatory drug (NSAID), meloxicam, can induce tolerant phenotypes during experimental infection. NSAIDs are broadly used in human and veterinary medicine to relieve pain and inflammation caused by noxious stimuli by inhibiting cyclo-oxygenase (COX), a family of enzymes responsible for prostaglandin synthesis and release in target tissues. COX-1 prostaglandins are protective, with constitutive expression in key tissues like the gastric mucosa, kidney, brain, and bloodstream that helps maintain normal physiology (Mitchell, Akarasereenont, Thiemermann, Flower and Vane, 1993). COX-2 prostaglandins are reactive and their expression is induced by inflammatory cytokines at the site of tissue injury (Mitchell *et al.*, 1993). They are responsible for signaling pain and further escalating the inflammatory response via recruitment of immune cells (Aoki and Narumiya, 2012). Accordingly, we use meloxicam in this study because it is a COX-2 specific inhibitor.

Using wild-caught, experimentally-infected house finches, we measured how NSAID therapy impacted disease tolerance. We predicted that daily meloxicam treatment would reduce clinical signs without changing the bacterial burden as compared to untreated, but infected, birds. We tracked eye pathology and pathogen load over twenty-eight days following inoculation with MG. We additionally measured antibody titers to better understand how NSAID treatment affects the activation of adaptive immunity. This study reflects outcomes from two separate trials, the first conducted in 2016 and then replicated in 2018 using the same methods but a unique cohort of hatch-year birds.

Methods

Capture Sites and Permits

House finches used in the first trial were captured between 5-15 September, 2016, using mist nets at private residences around Ames, Iowa, USA (42.0254675° N, 93.62688° W). House finches used in the second trial were captured between 23 June and 26 August, 2018 at two private residences in Ames using both mist nets and feeder traps. All procedures were approved under permits from the Iowa Department of Natural Resources (SC1133), the U.S. Geological Survey Bird Banding Lab (23952), the U.S. Fish and Wildlife Service (MB82600B), the Institutional Animal Care and Use Committee at Iowa State University (7-16-8311-Q; 6-17-8543-Q), and the Institutional Biosafety Committee at Iowa State University (Both experiments: 17-I-0021-A).

Initial Housing and Quarantine

At capture, birds in the first trial were assigned an eye score (0-3 per eye; Sydenstricker *et al*., 2006) to assess eye pathology consistent with mycoplasmal conjunctivitis. Eyes that scored "0" were clinically normal, those that scored "1" had mild inflammation, those that scored "2" had moderate inflammation often with conjunctival eversion, and those that scored "3" had severe inflammation with purulent discharge, crusting, and eye spasm. Birds scoring "0" in both eyes were considered eligible for this study and brought into captivity. Birds were dusted with GardenTech Sevin-5 Ready-To-Use 5% Dust (TechPac, LLC., Atlanta, GA) to remove any ectoparasites before entering the captive facility, where they were housed in individual cages (76 cm x 46 cm x 46 cm) with access to food and water ad libitum. Birds received a 50:50 mix of black oil sunflower seed and food pellets (Daily Maintenance Nibbles, Roudybush, Inc., Woodland, CA) throughout the study. Birds were started on a 12 hr Light: 12 hr Dark cycle for the first month in captivity, then transitioned to an 11 hr Light: 13 hr Dark cycle to mimic natural

conditions. Day length then remained constant throughout the duration of the study period, and rooms were maintained at 23°C.

Birds were monitored every three days for two weeks for clinical signs of disease. A blood sample was taken from the ulnar vein 14 days after capture to test for the presence of anti-MG IgY antibodies using enzyme-linked immunosorbent assay (ELISA, see details in "Sampling, polymerase chain reaction, and serology"). Briefly, the ulnar vein was punctured using a 26G needle and blood was collected in a heparinized microcapillary tube. Blood was then separated by centrifugation and plasma stored at -20°C until the ELISA. Individuals that remained nonclinical and had ELISA sample:positive (S/P) ratios < 0.0229 on light spectroscopy (Hawley, Grodio, Frasca, Kirkpatrick and Ley, 2011) were enrolled in this study and assigned an aluminum leg band with a unique numeric identifier. Birds that did not meet these criteria were released at their original site of capture.

Birds in the second trial were processed in the same manner as birds in the first trial, except where detailed below. Birds were maintained on a 12 hr Light: 12 hr Dark cycle throughout the duration of this experiment, with ad libitum access to water, grit, cuttlebone, and a 20:80 mix of black oil sunflower seeds and food pellets (Daily Maintenance Nibbles, Roudybush, Woodland, CA). Due to unexpected mortalities during other experiments in 2017, all birds received a prophylactic for trichomoniasis: 0.025% dimetridazole (Cankerex Plus at 0.25 g/L water; Medpet, Benrose, South Africa) in their drinking water for 5 days. Additionally, two birds received a prophylactic for coccidiosis: 2.5% toltrazuril (Endocox at 1.22 g/L water; Jedd's Fine Avian Products, Anaheim, CA) in their drinking water for 3 consecutive days for 3 weeks, followed by 3 days every 2 weeks until recruitment into this trial. Both birds also received probiotics in their drinking water once per week to regulate the intestinal microflora

(Bene-Bac Plus Bird and Reptile at 1 g/L water; PetAg, Inc., Hampshire, IL). Anticoccidial treatment was discontinued 15 days before NSAID treatment began, followed by probiotics 6 days prior, and these birds were assigned to opposite experimental groups.

NSAID and *Mycoplasma* treatments

Birds were randomly assigned to one of two treatments per trial: oral meloxicam (NSAID) or control (no NSAID). Treatment began one or two days prior to experimental inoculation with MG (Figure 1) to maximize the effect of prophylactic analgesia, the standard of care in pre-operative veterinary patients (Hawkins and Paul-Murphy, 2011). Birds in the first trial received either 1 mg/kg meloxicam plus water for a total fluid volume of 100 µL (n=8) or 100 µL water with no NSAIDs (n = 10) orally via micropipette each day. Birds in the second trial received either 1 mg/kg meloxicam (n = 8) or 2 µL/kg water (n = 8) for comparable volume orally via micropipette each day. This equated to 36-44 µL of fluid volume per treatment for birds of average weight (18-22 g). Dosages were adjusted once per week based on weight.

On 21 October 2016 and 14 September 2018 (experimental days 0), birds were inoculated with an expanded 7th generation in vitro passage of finch MG isolate 'VA1994' (stock ID 7994-1-7P 2/12/09; D. H. Ley, North Carolina State Univ., College of Veterinary Medicine, Raleigh) suspended in Frey's medium. A volume of 25 µL containing a total of 1.88 x 10^5 color-changing units (CCU) was applied to each eye via micropipette, and birds were maintained in horizontal recumbency until fluid dissipated below the eye rim.

Sampling, polymerase chain reaction, and serology

Eyes were scored three times per week using the same scale applied at initial capture, starting on day -1 (baseline, pre-inoculation, Figure 1) by a single observer who was blind to

treatments (J. Adelman). Conjunctival swabs were collected on days -1, 3, 7, 14, and 28 of infection to evaluate pathogen load (Figure 1). To do so, the ventral eyelid margin was abducted using sterile forceps, and a sterile cotton-tipped applicator dipped in tryptose phosphate buffer (TPB) was inserted and rotated for 5s. Swabs were then immersed in dedicated microcentrifuge tubes with 300 µL TPB, wrung out for 5s, and stored at -20°C.

We used the Qiagen DNeasy Blood and Tissue kit (Cat no. 69504/69506, Qiagen, Valencia, CA) to extract DNA from the swab samples suspended in TPB, followed by quantitative polymerase chain reaction (qPCR) targeting the *mgc2* gene, which encodes a well-described cytadhesin-like protein, using previously published conditions and primers (Grodio, Dhondt, O'Connell and Schat, 2008). Blood samples were taken, as described above, on days 14 and 28 of infection (Figure 1). Samples were immediately centrifuged to separate the plasma, then stored at -20°C for later serological evaluation using a commercially available ELISA kit, adapted for use in finches (cat # 99-06729, IDEXX Laboratories, Inc., Westbrook, ME; validated in Grodio, Buckles and Schat, 2009). Of note, plasma samples were screened in duplicate and retested, as volume would allow, when their coefficient of variation (CV) exceeded 10%. If the sample's CV on the rerun was <10%, that S/P ratio was used, otherwise the S/P ratios were averaged between original and reruns. Additionally, the blanks on one ELISA plate had negative absorbances resulting in some samples having inflated S/P ratios, calculated as [mean sample absorbance − mean blank absorbance]/[mean positive control absorbance − mean blank absorbance]. However, the trends were qualitatively the same when these samples were omitted (see Appendix).

Data Analysis

Data on eye score, pathogen load, and antibody titers were analyzed with linear mixed-effects models in R, version 3.5.2 (R Development Core Team, 2018) using packages nlme and emmeans (Pinheiro, 2000; Lenth, 2021). The models included day post-inoculation (DPI), treatment-year, and their interaction as fixed effects, as well as bird ID as a random effect. Eye pathology was measured as the sum of left and right eye scores per bird per day. Pathogen load was measured as the number of *mgc2* copies detected per eye per day, which was then summed by individual and $\log_{10}$ transformed to control for non-normality. Antibody titer was again measured as the ratio of sample absorbance to positive control absorbance from ELISA. All models included an AR1 autocorrelation function to control for temporal autocorrelation. Finally, we evaluated differences in clinical outcome between first-trial treated and control birds, second-trial treated and control birds, and control birds across trials, using post-hoc tests with an adjusted p-value based on a multivariate *t* distribution (lsmeans and contrast functions in emmeans; Lenth, 2021).

Quantifying Tolerance

Point tolerance offers a snapshot of disease severity by comparing peak host pathology to peak pathogen burden (Little, Shuker, Colegrave, Day and Graham, 2010). We fit a linear mixed-effects model for pathology using pathogen load, treatment-year, and their interaction as fixed effects with a random effect for bird ID, using pre-infection data (0 pathology, 0 pathogen load) and peak-infection data to compare tolerance slopes. By this method, shallower slopes indicated higher point tolerance, which we evaluated using the specific pairwise comparisons as described above.

As an alternative to point tolerance, we created a metric to yield a more continuous range of values for pathology and pathogen load, akin to range tolerance (Little *et al.*, 2010).

Specifically, we calculated discrete integrals of pathogen load (log_{10}) and pathology over time for each individual. We estimated the area under each curve using a series of polygons for which the width equaled the number of days between samples and the height was assumed to change linearly between each time point. Because we collected eye scores three times per week and pathogen load only once per week, this method generated a more fine-scale approximation for pathology (9 polygons) than pathogen load (3 polygons). Of note, we excluded days 0-3 from the integrals of pathogen load as we did not collect conjunctival swabs immediately after inoculation and so did not have a value for pathogen load on day 0. We then fit a linear mixed-effects model predicting the integral of pathology using the integral of pathogen load (log_{10}), treatment-year, and their interaction as fixed effects and bird ID as a random effect. This dataset included pre-infection integrals (0 units for all birds) and during-infection integrals for each bird. We found that squaring the integral of pathogen load improved model fit by over 2 units of Akaike's Information Criterion for small sample sizes (AICc) (Burnham and Anderson, 2002), and so the final model included the squared integral of pathogen load. As with point tolerance, shallower slopes indicated higher range tolerance over time, which we evaluated using the same pairwise comparisons as above.

Results

Pathology

Eye pathology, measured by the sum of left and right eye scores, showed patterns typical of MG infection, increasing and then resolving over the course of 28 days (Figure 2*a*, LMM: DPI: $F_{9,270} = 22.90$, $p < 0.001$). Pathology differed by treatment and trial (LMM: treatment-year: $F_{3,30} = 4.60$, $p = 0.01$; treatment-year x DPI: $F_{27,270} = 2.04$, $p = 0.002$) and was notably most severe in first-trial control birds, which reached peak-pathology on day 14 post-inoculation with

a more than two-fold higher eye score than any other group. Based on Tukey pairwise comparisons, pathology differed by treatment in the first trial (days 10-17, $p \leq 0.02$), but not the second (all days, $p \geq 0.6$). Further, while meloxicam-treated birds developed similar pathology between trials, second-trial control birds showed about half the pathology of first-trial controls (days 5-24, $p \leq 0.05$).

Pathogen Load

The $\log_{10}$ values of pathogen load, summed across both eyes, showed a peak after experimental infection followed by a gradual decline over the study period (Figure 2*b*, LMM: DPI: $F_{4,120} = 317.73$, $p < 0.001$). All treatment groups peaked at above 10^5 copies of MG DNA (Figure 2*b*), and pathogen load did not markedly differ across treatment or trial (LMM: treatment-year: $F_{3,30} = 1.79$, $p = 0.17$; treatment-year x DPI: $F_{12,120} = 1.19$, $p = 0.30$). That being said, first-trial control birds maintained the highest pathogen loads and second-trial control birds maintained the lowest pathogen loads, significantly diverging from one another on days 14 and 28 ($p < 0.02$). By contrast, meloxicam-treated birds showed nearly uniform pathogen loads between trial years and did not diverge from control birds within either year (all $p > 0.2$, except second-trial birds on day 28 $p = 0.08$).

Point Tolerance

We evaluated point tolerance using a linear function by which peak pathology varied by the $\log_{10}$ of peak pathogen load (Figure 3*a*; LMM: peak pathogen load: $F_{1,30} = 241.29$, $p < 0.001$). Tolerance slopes differed by treatment and trial, with first-trial control birds showing the lowest tolerance (steepest slope) (LMM: treatment-year: $F_{3,30} = 8.39$, $p < 0.001$, treatment-year x peak pathogen load: $F_{3,30} = 7.59$, $p < 0.001$). During the first trial, control birds had steeper

slopes of eye score by pathogen load (LMM estimate = 0.81) than meloxicam-treated birds (LMM estimate = 0.55; Figure 3*a*; adjusted $p = 0.05$). However, in the second trial, control birds had comparable slopes (LMM estimate = 0.47) to meloxicam-treated birds (LMM estimate = 0.36; Figure 3*a*; adjusted $p = 0.65$). Thus, the different patterns between trials may actually reflect differences in baseline tolerance, as first-trial control birds had lower point tolerance than second-trial controls (Figure 3*a*, adjusted $p = 0.006$).

Range Tolerance

We evaluated range tolerance using a quadratic function whereby the integral of pathology varied by the square of the integral of $\log_{10}$ pathogen load (Figure 3*b*; LMM: pathogen load integral^2: $F_{1,30} = 208.94$, $p < 0.001$). Again, tolerance slopes differed by treatment and trial, with first-trial control birds showing the least tolerance over time and second-trial meloxicam-treated birds showing the most tolerance over time (LMM: treatment-year: $F_{3,30} = 5.17$, $p = 0.005$; treatment-year x pathogen load integral^2: $F_{3,30} = 7.64$, $p < 0.001$). During the first trial, control birds had steeper slopes of eye score by pathogen load integral^2 (LMM estimate = 5.4 x 10^{-3}) than meloxicam-treated birds (LMM estimate = 3.3 x 10^{-3}; Figure 3*b*; adjusted $p = 0.02$). However, in the second trial, we found no evidence for different slopes between control (LMM estimate = 3.3 x 10^{-3}) and meloxicam-treated birds (LMM estimate = 2.3 x 10^{-3}; Figure 3*b*; adjusted $p = 0.59$). As with point tolerance, higher baseline tolerance among birds in the second trial likely contributed to these patterns, although the difference in control birds' range tolerance was less pronounced across trials (Figure 3*b*, adjusted $p = 0.08$).

Antibody Response

All birds started with negligible anti-MG IgY antibody titers, consistent with no prior pathogen exposure. Titers then peaked around day 14 and fell as birds recovered (Figure 4, LMM: DPI: $F_{2,60} = 58.09$, $p < 0.001$). Overall, titers did not differ substantially by treatment or trial (LMM: treatment-year: $F_{3,30} = 1.81$, $p = 0.17$; treatment-year x DPI: $F_{6,60} = 1.34$, $p = 0.25$), though meloxicam-treated birds peaked below control birds in the first trial ($p = 0.02$).

Discussion

The current studies used chemotherapeutics to evaluate whether modulating the pro-inflammatory response to infection could enhance host tolerance in a model wildlife disease system, finch mycoplasmosis. We hypothesized that exogenous treatment with non-steroidal anti-inflammatory drugs (NSAIDs) would significantly reduce conjunctival pathology, shifting the host response toward tolerance (reduced pathology for a given pathogen load). Though birds reached similar peak pathogen loads across trials, NSAID treatment produced mixed results, with treated birds developing milder eye lesions during the first trial but not the second. Thus, clinical outcome appeared to depend more on trial than treatment due to differences in baseline tolerance between study years, complicating the interpretation of NSAID effects.

Our study sought to use NSAIDs to damp down the clinical signs of mycoplasmal conjunctivitis. However, in a population with high baseline tolerance, which would show low severity of clinical signs on average, the margin to reduce pathology and thus tease out an effect of treatment from the noise of phenotypic variation shrinks. This provides one explanation why the effect of NSAID treatment was apparent in the first trial, but not the second. Second-trial control birds showed peaks in pathology that were already half that of first-trial controls and as such, may have reached a point beyond which NSAID treatment would further reduce clinical signs. Indeed, control birds in the second trial present much like NSAID-treated birds in both

trials.

Given the phenotypic variation we observed across trials, future work could manipulate certain aspects of experimental design to better assess the role of pro-inflammatory pathways in disease tolerance. We targeted the COX pathway in these studies using a fairly conservative NSAID dosing regimen. Though higher or more frequent doses of meloxicam have been reported in the literature, they have not been evaluated for long-term treatment up to several weeks duration (Sinclair *et al*., 2012; Dijkstra *et al*., 2014). Miller, Hill, Carrasco, and Patterson (2019) additionally found that plasma concentrations dropped below therapeutic targets within 12 hours in zebra finches treated with 1 or 2 mg/kg meloxicam intramuscularly, suggesting that our NSAID treatment may have only exerted any effect on inflammation half of the time. However, we must also consider how this might increase handling time and stress, which could in itself change disease outcomes without developing more discrete methods of delivery, such as medicated water. Regardless of approach, increasing the efficacy of treatment could better control for individual variation but perhaps still not overcome population-level swings in disease tolerance. As such, replicating this study in a population with low standing levels of tolerance (e.g., a naïve population or one in which MG has been established for a shorter time, Adelman *et al.*, 2013, Bonneaud *et al.*, 2019) may better control for this inter-trial noise and allow for a more sensitive evaluation of the role of the COX pathway in disease tolerance.

Additionally, there may be other chemotherapeutic regimes that can more effectively modulate disease tolerance. For instance, corticosteroids dampen inflammation by acting on molecules further upstream of those targeted by NSAIDs (Clark-Price, 2013). While there is some evidence that pre-infection baseline corticosterone levels and subsequent conjunctivitis negatively correlate in house finches (Adelman, Moore, and Hawley, 2015), this relationship

varies by sex (Love, Foltz, Adelman, Moore, and Hawley, 2016), suggesting a complex role for the hormone in MG pathology. Moreover, because of their broad, systemic effects on host physiology, interpreting the effects of corticosteroid treatment on infection can prove challenging. Rather than suppressing prostaglandin synthesis to induce tolerance, future studies might leverage the natural variation in host tolerance to measure prostaglandin production and help elucidate the role of COX pathways in disease tolerance.

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Figure Legends

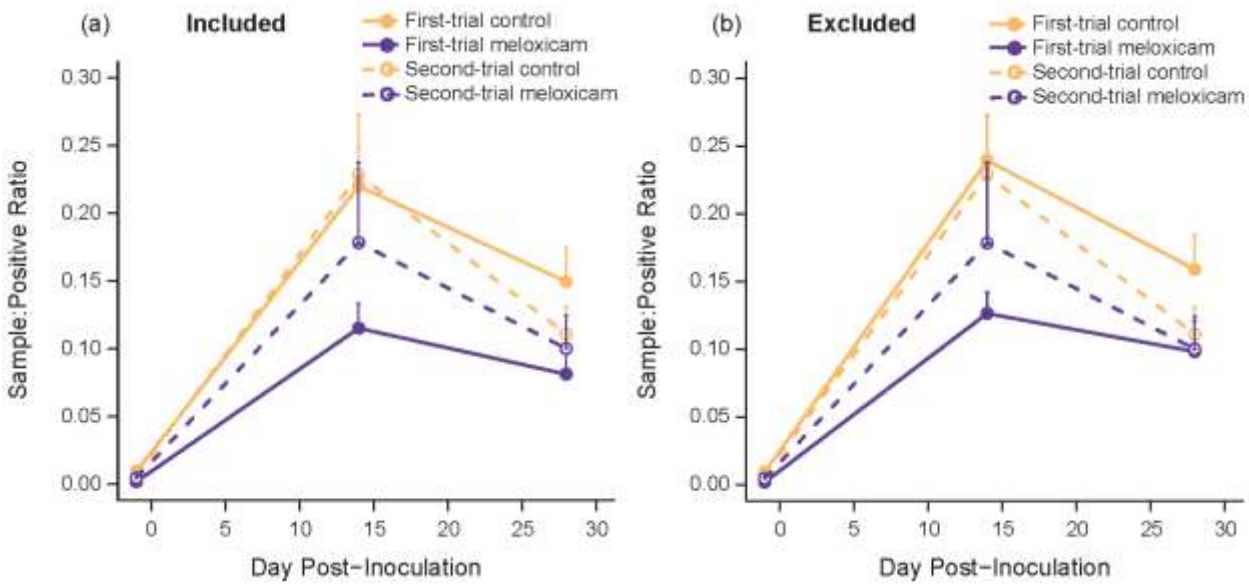
Figure 1 Timeline for both trials included in this study. Treatment with an NSAID or water began 1-2 days prior to experimental inoculation with *Mycoplasma gallisepticum* (MG) on day 0. The sampling regime included collecting eye scores to measure pathology, conjunctival swabs to measure pathogen load, and blood samples to measure MG-specific antibody titers.

Figure 2 NSAID treatment significantly reduced the severity of conjunctivitis (a) in the first trial but not the second. However, with second-trial control birds only developing about half the pathology of first-trial controls, there was a narrower margin for us to measure a treatment effect. Though NSAID treatment had no effect on pathogen load (b) within trials, first-trial control birds had significantly higher pathogen loads than second-trial control birds from day 14 on. Points show group means plus 1 standard error.

Figure 3 NSAID-treated birds showed higher point tolerance (a) than control birds during the first trial but not the second. Similarly, NSAID-treated birds showed higher range tolerance (b) than control birds in only the first trial. Given that second-trial control birds also showed higher tolerance than first-trial controls, differences in baseline tolerance within the source population across years may actually be masking a consistent treatment effect. Points on both plots represent individual birds, with the lines or curves reflecting the fixed effect estimates for each group from the linear mixed-effects models.

Figure 4 NSAID treatment significantly reduced peak (day 14) antibody titers in the first trial but not the second. Points show group means plus 1 standard error.

Appendix



The blank wells used to standardize sample:positive ratios had negative absorbances on one enzyme-linked immunosorbent assay plate, leading to somewhat inflated values (a). When

plasma samples tested on this plate were removed (b), treatment and trial continued to have a negligible effect on titer ($LMM_{included}$: treatment-year: $F_{3,30} = 1.81$, $p = 0.17$; treatment-year x DPI: $F_{6,60} = 1.34$, $p = 0.25$; $LMM_{excluded}$: treatment-year: $F_{3,30} = 1.67$, $p = 0.19$; treatment-year x DPI: $F_{6,54} = 1.26$, $p = 0.29$).