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American Goldfinches (Spinus tristis): Changes in Behavior During Mycoplasma gallisepticum Infection

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AMERICAN GOLDFINCHES (*SPINUS TRISTIS*): CHANGES IN BEHAVIOR DURING *MYCOPLASMA GALLISEPTICUM* INFECTION

By

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ABSTRACT

American Goldfinches (*Spinus tristis*): Changes in Behavior During *Mycoplasma gallisepticum* Infection

(Under the direction of Dr. Susan Balenger)

Animal sickness behavior is an important component of disease ecology and is essential to understanding wildlife diseases and how and where animals allocate resources for survival. This study examines sickness behaviors, the extent of conjunctivitis, and the presence of an antibody response in relation to a *Mycoplasma gallisepticum* (MG) respiratory infection in American goldfinches. We conducted an experimental infection of American goldfinches and recorded behavior videos, and collected blood samples, throat swabs, eye swelling, and mass data at multiple time points throughout the experiment. An ELISA-serum assay was run after the conclusion of the study to identify the presence of MG-specific antibodies in each bird's serum. Our results showed an increase in eye swelling and stationary behaviors of infected goldfinches and a decrease in mass and active behaviors during the late stage of the experiment. The ELISA assay showed only 71% (5/7) of American goldfinches seroconverted by the end of the experiment. These findings suggested that American goldfinches are affected by individual variation in generating an immune response to MG, compared to house finches, and can further our understanding of how behavioral responses relate to disease progression.

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INTRODUCTION

In terms of disease ecology, sickness behavior is the behavioral changes induced by an animal's immune response (Panzera 2013). Often, an animal's behavioral and physiological responses change to show symptoms of infection as a response due to disruptions in homeostasis, negatively affecting their health (Panzera 2013). Behaviors are a byproduct of infection and are an adapted part of the host's defense mechanism (Love et al., 2016; Hawley et al., 2006). Compared to healthy animals, sick animals behave differently, including changes in social behavior (Lopes et al., 2021). Some of these behaviors inherently promote the spread of disease, such as infected animals drinking water and contaminating the shared resource (Faustino et al., 2004; Stallknecht et al., 1998; Lopes et al., 2021). As a result, other animals within the same species risk exposure to the pathogen and its infectious state (Faustino et al., 2004). Sickness behaviors vary among diverse vertebrate groups and species (Lopes et al., 2021). In birds, sickness behaviors encourage survival by reducing physical activity to conserve energy to fight infection (Love et al., 2016; Adelman et al., 2017; Bouwman and Hawley 2010; Lopes et al., 2021). Their reduced energy for physical strength causes a decrease in food intake, leading to significant mass loss (Bonneaud et al., 2003). As a result of the acute phase of the immune response, reduced ability to forage is another component that causes significant mass loss (Moyers et al., 2015). The immune response challenges the bird's energy needs, resulting in lower aggression levels, metabolic changes, and activity changes (Moyers et al., 2015).

Our understanding of diseases benefits from incorporating the study of animal behavior. Levitis et al. (2009) proposed their own definition of behavior as an organism's internal responses to stimuli, excluding any changes occurring during developmental stages. When an animal is stimulated either internally or externally, its brain processes that stimulatory information and produces a reaction to cope with that stressor (Levitis et al., 2009). When an animal falls ill from a pathogen, its immune system triggers a biological response to fight the infection (Panzera 2013). The infection uses up the animal's energy sources as its body tries to survive and return to its healthy, homeostatic state (Panzera 2013). Also, the pathogen targets specific tissues, resulting in changes in the tissue structure and function (Marshall et al., 2018). An active immune response allocates the animal's energy to fight the infection (Marshall et al., 2018). This results in a physical reaction characterized by inflammation and heightened symptoms resulting from infection (Marshall et al., 2018). Consequently, the animal's behavior is likely to change when infected (Panzera 2013). Disease ecology utilizes a reciprocal feedback mechanism correlating with animal behavior (Ezenwa et al., 2016; Panzera 2013). Animal sickness behaviors vary based on the pathogen and species, depending on individual and environmental variables (Lopes et al., 2021; Ezenwa et al., 2016). A negative feedback loop facilitates the elimination of either the pathogen or the host (Ezenwa et al., 2016). Therefore, pathogens trigger infectious diseases and induce a behavioral response in the infected animal as the disease progresses (Adelman et al., 2017; Moyers et al., 2015; Bouwman and Hawley 2010; Ezenwa et al., 2016). Regarding host survival and transmission of a pathogen, behavior is an important indicator of ecological and evolutionary effects on different species (Lopes et al., 2021).

This study was designed to understand how sickness behaviors in the American goldfinch relate to a respiratory infection. The pathogen used to study disease progression is the avian pathogen *Mycoplasma gallisepticum* (MG). MG is a bacterial pathogen that arose in poultry and developed a lineage that is now known to affect house finches (Ley 2008). This spherical-shaped bacterium has a plasma membrane that adheres to host cells, transports nutrients, and displays antigenic variation on its surface (Ley 2008). MG surface antigens present adhesin and hemagglutinin-like proteins or lipoproteins (Evans et al., 2005; Ley 2008). According to Ley 2008, the infectivity of the MG strain depends on the isolate's genotype and phenotype, propagation, the passage number of subculture, and challenge route and dosage. The clinical symptoms indicate how well the immune system responds to MG infection instead of the pathogen's direct effects (Vinkler et al., 2018). One week after an experimental MG infection in chickens, there is a considerable presence of proinflammatory cytokines and immunoglobulins, which causes inflammation (Ali and Ali 2019). In chickens, it is known as chronic respiratory disease, characterized by abnormal breathing sounds, coughing, nasal discharge, and conjunctivitis (Evans et al., 2005; Ley 2008).

Transmission studies in house finches show feeding behavior through foraging and at feeders increases the risk of MG pathogen exposure (Adelman et al., 2015; Dhondt et al., 2007). In the wild, horizontal transmission occurs within the same flock of birds through droplets entering the upper respiratory tract or conjunctiva directly or indirectly (Ley 2008; Faustino et al., 2004). Vertical transmission occurs directly between generations, such as when infected mother hens lay eggs (Ley 2008; Faustino et al., 2004). The offspring hatches and spreads the disease throughout the flock (Ley 2008). MG-infected house finches flock toward feeders as an

easy food source (Adelman et al., 2015). Therefore, the feeders serve as a contamination site through horizontal transmission (Moyers et al., 2018; Faustino et al., 2004). This change in feeding behavior increases the chance of acquiring MG infection in the wild (Adelman et al., 2015; Moyers et al., 2018). Studies performed by Dhondt et al. (2007) show that feeders are the primary way for the MG pathogen to spread to naive house finches (Dhondt et al., 2007; Adelman et al., 2015; Moyers et al., 2018). Those infected birds develop antibiotic resistance to the bacterium (Bonneaud et al., 2012). The finches gain immunity as their immune systems evolve to combat future infections or mutated strains (Bonneaud et al., 2012; Hochachka et al., 2021). Similarly, the pathogen evolves to become more infectious (Hochachka et al., 2021). Finches with newly acquired immunity eventually become resistant to more minor infectious strains (Bonneaud et al., 2012; Hochachka et al., 2021). Naive finches and finches exposed to less virulent strains are susceptible to severe disease (Fleming-Davies et al., 2018; Dhondt et al., 2017). The MG bacteria becomes more virulent in North America because the host's immunity passes through generations, indicating a genetic component to immunity (Dhondt et al., 2017; Hochachka et al., 2021). Virulent strains are evolving to successfully infect hosts with acquired resistance and produce greater acquired immunity in recovered hosts (Dhondt et al., 2017; Hochachka et al., 2021).

The acute immune response influences house finch behavior through physical symptoms, such as conjunctivitis (Moyers et al., 2015). Conjunctivitis is the inflammation and swelling of the mucosal surface of the eye conjunctiva, causing impaired vision (Dhondt et al., 2007). The physical inflammatory swelling in their eyeballs changes the bird's behavior through its inability to see its surroundings (Adelman et al., 2017). Their movement is limited from the

inability to reach food sources or remain near feeders, contributing to mass loss and minimal movement as sickness behavior (Adelman et al., 2017). Their sickness behaviors are a negative consequence of inflammation (Bouwman and Hawley 2010; Adelman et al., 2013). To fight off the infection, respiratory tract antibodies are produced in response to MG infection (Ley 2008). By measuring antibody levels, we can understand how quickly and strongly the finch reacted to MG inoculation (Ley 2008).

MG strains in North America were introduced by wild birds that are known MG reservoirs from other regions globally, primarily affecting North American house finches (Dhondt et al., 2014). Strains of MG have diverged to affect a wide range of wild birds, including American goldfinches, and became an epidemic in American house finches during the winter of 1993-94 (Fischer et al., 1997). The American goldfinch (*Spinus tristis*) is a North American songbird, known as a new member of the Fringillidae (or New World Finch) family. Goldfinches are diurnal, social birds that are active throughout the day (McGraw and Middleton 2020). They live in temperate areas in the wild, where they flock together and share bird feeders (McGraw and Middleton 2020). They move in a fluid and mobile manner and fly with quick, hovering beats (McGraw and Middleton 2020). Their only mode of mobility on the ground is walking (McGraw and Middleton 2020). During this movement, a goldfinch uses its beak to scratch and reaches its feathers (McGraw and Middleton 2020). One study found that preening is their most common behavior in captivity, with 17% of the six-month time spent under observation (Coutlee 1963). It occurs the most during the molting period after the bird reaches maturity (McGraw and Middleton 2020; Coutlee 1963). Other infections, such as coccidial infection, decrease social behavior but do not significantly affect preening (Surmacki and Hill 2014). Goldfinches are late

breeders, only reproducing in the summer months of June and July (McGraw and Middleton 2020). This is most likely due to less predation pressure and higher seed availability to feed their young (McGraw and Middleton 2020). They also exhibit greater aggressive behavior during this time (Popp 1988). Because they are granivores, their diet is exclusively seeds, preferring sunflower, thistle, and elm seeds (McGraw and Middleton 2020). They are daytime feeders and are known to adjust to best reach their food, mainly using their feet, especially when hanging upside down (McGraw and Middleton 2020; Coutlee 1963). After feeding, goldfinches usually return to their perch, fluff and shake their feathers, or clean their bills using their wings (Coutlee 1963). As discussed by Coutlee 1963, understanding baseline maintenance behavior helps determine what behavioral changes have occurred throughout generations.

Sickness behavior is thought to be a consequence of an energetic trade-off with investment in immunity (Bonneaud et al., 2003). Bonneaud et al. (2003) proposed that sickness behaviors encouraged survival by reducing activity to conserve energy to fight off infection. Infected birds are more vulnerable to starvation, predation, and secondary infections due to MG infection, which causes reduced vision and suppresses their immune system (Williams et al., 2014; Faustino et al., 2004). MG-infected finches exhibit sickness behavior through low aggression, lethargy, mass loss, decreased social behavior, reduced locomotion, and less water intake (Bouwman and Hawley 2010; Moyers et al., 2015; Adelman et al., 2013). Immobility, lethargy, and low motivation to engage in social behaviors were suggested by multiple studies to affect the bird's ability to detect and escape from predators (Adelman et al., 2017; Bouwman and Hawley 2010; Adelman et al., 2013). The severity of MG-induced conjunctivitis limited the bird's ability to see, increasing their likelihood to be susceptible to predation and capture

(Adelman et al., 2013). An inevitable result of infection was the cause of the significant decrease in anti-predator behaviors (Adelman et al., 2013).

Despite the ongoing infection, behavioral tolerance to MG shows physical proof of normal behavior (Balenger, unpubl. data). In Eastern Bluebirds, mass loss was the only difference between control and infected birds (Balenger, unpubl. data). They did not exhibit any sickness behaviors, indicating this bird species has high behavioral tolerance to MG (Balenger, unpubl. data). Not all animals within the same species exhibit the same sickness behaviors or clinical symptoms (Lopes et al., 2021; Farmer et al., 2005). Compared to Eastern Bluebirds, house finches also serve as reservoirs for MG, which can transmit the pathogen to American goldfinches (Dhondt et al., 2013; Farmer et al., 2005; Faustino et al., 2004).

American goldfinches that interact with MG-infected house finches also contract the pathogen through experimental methods but are not effective transmission sources in the wild (Farmer et al., 2005). American goldfinches had a lower infection incidence than house finches (Ley 2008). For house finches, American goldfinches, and evening and pine grosbeak, the symptoms of MG infection include severe conjunctivitis, indicating that these hosts serve as reservoirs for MG (Farmer et al., 2005; Dhondt et al., 2013). Bird species, including Carolina chickadees, tufted titmouse, and Eastern Bluebird, did not exhibit significant differences between control and infected birds (Balenger, unpubl. data). Eastern Bluebirds lack the observable physical symptom of conjunctivitis after MG infection (Balenger, unpubl. data). Still, infection with MG has a physiological and survival cost for them, as they serve as host reservoirs for the pathogen (Balenger, unpubl. data). Other studies observed similar clinical symptoms involving different hosts, such as chickens and turkeys (Evans et al., 2005; Ley 2008). Although it mainly

shows infectiousness in the respiratory tract and conjunctiva, MG infection can spread to other organs, including the brain (Ley 2008). Infected chickens and turkeys demonstrate thicker mucous membranes throughout their respiratory system, swollen epithelial cells, and destroyed cilia in the trachea (Evans et al., 2005; Ley 2008).

In this experiment, I asked if the prevalence of symptoms and changes in behavior are altered in relation to disease progression. I wanted to examine when and to what extent conjunctivitis develops among MG-infected American goldfinches by gathering eye scores throughout the experiment. I wanted to determine if American goldfinches exhibit sickness behaviors after an MG infection by recording behavior videos. I wanted to determine whether infected American goldfinches develop an antibody response following infection with MG. I used ELISA assays to quantify infection prevalence via the presence of antibodies. I predicted that MG infection reduces behavioral responses. If antibodies have developed, I predicted that the individual birds are overcoming the infection and getting better. Antibodies could represent either the best or worst condition of the bird, indicating its response to MG infection. Sickness behaviors resulting from infectious diseases can be a result of individual variation. The MG infection could cause weight loss (Adelman et al., 2013; Adelman et al., 2017; Bonneaud et al., 2012; Bonneaud et al., 2003; Davis et al., 2004). Weight loss occurs in birds who eat less food, and as a result, they have less energy to engage in activities (Adelman et al., 2017; Bouwman and Hawley 2010; Adelman et al., 2013). Overall, sickness behaviors are potential result of tradeoff in order to upregulate the immune response.

MATERIALS AND METHODS

Thirteen American goldfinches (*Spinus tristis*) and one house finch (*Haemorhous mexicanus*) were captured using a mist-net during the winter months of January and February 2021 in Oxford, Mississippi. When caught, their weight and gender were recorded and banded by color. These birds were transferred to and housed in the aviary at the University of Mississippi Field Station (Abbeville, MS, USA). The American goldfinches were housed in cages, sized 51 cm high x 51 cm wide x 51 cm long. The house finch lived a cage without dividers, sized 51 cm high x 51 cm wide x 102 cm long. A cage rack was separated by a divider, forming two individual side-by-side cages. Three cage racks were stacked vertically. Cage paper lined the bottom of each cage and was changed weekly while performing bird husbandry. The fourteen birds were given food, water, and tree branches as perches. Each cage contained three various slim alder and elm branches taken from the Field Station landscape. Their feet easily gripped the branch and allowed movement between branches and a place to perch. Their diet consisted of sunflower seed mix in the left dish and water mixed with a drop of Vita-Sol in the right dish *ad libitum*. Six goldfinches were randomly chosen as controls and housed in a separate building at the Field Station. The remaining seven goldfinches and one house finch were inoculated with the same dose of MG. The house finch was included in this study to serve as a positive control of successful inoculation of MG. Bird husbandry was regularly performed, as well as monitoring room temperature and birds' physical activity to ensure good health. The goldfinches remained in captivity for six months since we were waiting for the molting period to end. We waited to

perform the experiment after the molt because it serves as an additional stressor along with the MG infection. All animal protocols and procedures for bird care were approved by Institutional Animal Care and Use Committee (IACUC) protocol #20-013.

All fourteen birds were quarantined for at least four weeks. We tested these birds at capture for antibodies using serum plate agglutination (SPA) tests. The SPA tests were conducted within two weeks of capture, then again before the experiment started. Only birds that tested negative were included in the study. Four days prior to inoculation (D-4), blood samples, mass, hemoglobin, and throat swabs were collected. Mass and hemoglobin data was analyzed on the same day it was collected. One day prior to inoculation (D-1), behavior videos were recorded to assess baseline behavior. Observing common behaviors pre-exposure helps determine typical healthy behaviors. The infected birds were inoculated with MG on D0 by eye droplet administration. The control birds had a sham inoculation with sterile culture media. Treatments were administered equally among all birds. Blood samples, mass, hemoglobin, and throat swabs were collected on day 2, 6, and 13 after initial inoculation (D2, D6, and D13 respectively hereafter) from both control and infected birds. *Pre* refers to four days before the start of the experiment. *Early* refers to two days after inoculation. *Mid* refers to six days after inoculation. *Late* refers to thirteen days after inoculation. We quantified the immune responses using the blood samples. Their blood samples were immediately centrifuged to separate serum from other blood components. During the study, serum was collected that we tested on D-4 and D13 between two and three weeks prior to the start of the experiment by SPA on May 29, June 5, June 6, and June 7. Hemoglobin was measured using a HemoCue Hb 201+ by drawing blood from the brachial wing vein onto a capillary cuvette. On D-4, D0, D2, D6, and D13, and D14, eye score

data was gathered on a scale of 0 to 3. All birds were given eye scores based on the progression of conjunctivitis, which is a symptom of MG during infection (Fischer et al., 1997). Mean eye score indicated the level of symptoms for each bird (Fischer et al., 1997). An eye score of 0 indicates no conjunctivitis, while a score of 3 indicates severe conjunctivitis (Fischer et al., 1997). All experimental data was collected between June 21, 2021 and July 10, 2021.

Behavior data was collected via video recordings at D-1, D5, and D12 of the experimental period. *Pre* refers to one day before the start of the experiment. *Mid* refers to five days after inoculation. *Late* refers to twelve days after inoculation. Each video was recorded in the morning between the times of 6:19 AM and 9:34 AM CDT. Room temperature in Fahrenheit and humidity percentage were also recorded at the beginning of the videos. Data collection began at five minutes and ended at twenty-five minutes of the video, totaling to twenty minutes of recorded data on each bird. This time period was chosen to lower the effects of human disturbance during camera set-up, which could cause an increase in each bird's excitement. Behavior was measured through quantifying the number of activities, including perching, preening, climbing/clinging, walking, jumps, hops, eating, and drinking. Perching, preening, climbing/clinging, and walking were counted individually in terms of time in seconds spent performing each activity using a stopwatch. Perching behavior was defined as time (sec) the bird sits on a branch/dish without performing any other listed activity. Preening was the time (sec) a bird picks at or cleans itself. Climbing/clinging was the time (sec) a bird spends on the cage bars. Walking was the time (sec) a bird walks along the branch. Jumps, hops, eating, and drinking were tallied individually as a number of events through the duration of the video using a tally counter. Jumps were the number of times a bird jumps and lands on different surfaces. For

example, a bird moving from branch to branch, branch to cage, cage side to cage side, dish to branch, or dish to cage. Hops were the number of times a bird jumps and lands on the same surface (i.e. branch). Eating was the number of times a bird landed on the food dish located on the left and picked seed from it. Drinking was the number of times a bird landed on the water dish located on the right and drank from it. Eating and drinking count does not include the number of pecks/sips the bird took from the dish when it landed once. The same researcher recorded and watched all videos and tallied behavior to eliminate variability. One video recording (D-1-SB513 MG) was not completed due to full camera storage but was re-recorded at 2:07 PM.

ELISA-Serum Protocol

An ELISA serum assay was performed using the IDEXX ELISA kit to determine the presence of MG-specific antibodies on D-4 and D14 for both control and infected birds. The wells of the ELISA plate were pre-coated with a fixed MG-specific antibody. Fresh blocking buffer of 20 mL was prepared using 18 mL of phosphate-buffered saline (PBS) and 2 mL of bovine serum albumin (BSA). Blocking buffer of 300 µL was added to all wells of ELISA plate and incubated for 40 minutes. The solution was decanted, then the plate was washed with 300 µL of wash solution three times. The wash solution was made of 475 mL of 1X PBS and 25 mL of 20X Tween 20. Tubes were prepared, containing diluent and serum from each bird at D-4 and D14 time points. Around 4.5 µL of serum was added to each tube, except for D14-SB519 MG and Pre-SB520MG. D14-SB519 MG only had 3 µL of serum. Pre-SB520MG only had 1 µL of serum. Pre-samples that were missing were SB522, SB524, and SB525, which serve as blanks on the plate. Positive and negative controls were supplied by the IDEXX ELISA kit. The controls

already contained diluent; however, diluent was added to these tube samples by accident. The serum samples were added to the wells from the respective tubes and incubated for 1 hour. The solution was decanted and washed three times with 300 µL of wash solution. Conjugate of 100 µL was added to the wells and incubated for 1 hour. Then, the solution was decanted and washed three times with 300 µL of wash solution. Substrate of 100 µL was added into each well and incubated for 15 minutes. The solution was not decanted, and 100 µL of stop solution was added to each well. The presence of blue colored solution in the wells was a visual indication that the serum contained MG-specific antigens. For numerical values, the BioTek plate reader reads the ELISA plate at absorbance value of 630 nm. The cutoff value (0.072) was determined as 2.5 standard deviations above the mean ELISA values for birds testing negative for MG by qPCR during the quarantine period (Hawley et al., 2011).

Analysis

All statistical analyses were performed using SAS 9.4 (SAS Institute, Cary, NC). We constructed separate repeated measures multivariate ANOVAS (PROC MIXED) with compound symmetry covariance structure to specifically examine the effect of MG infection on mass, circulating hemoglobin concentration, and behaviors associated with sickness over the course of the infestation. Treatment group and time point relative to inoculation (*Pre*, *Early*, *Mid*, and *Late*) and all interaction terms were included in the model as fixed effects. Overall significance of groups (treatment and time point relative to inoculation) was first evaluated using Type 3 tests. We then determined differences among groups at each level using least squares means with the slice option for any term that was significant in the full model.

RESULTS

Of the *Pre*, *Early*, *Mid*, and *Late* periods, the eight goldfinches infected with MG exhibited a significant decrease in mass in the *Late* period (Table 2, p=0.05). Mass did not significantly change for control birds, but was significantly less for birds experimentally inoculated with MG for two weeks (Figure 1). When looking at the infection results by day, the only variables that produced a significant decrease were mass and preening (Table 1). The effect of treatment, day, and interaction of treatment by day all showed significant increase in perching behavior (Table 1 and Figure 4). The most common period that produced significant effects on all measured variables was during the *Late* period, excluding hemoglobin, walking, and eating (Table 2). Under multiple ANOVA analyses, hemoglobin did not yield a significant difference (Tables 1 and 2). Jumping, hopping, eating, and drinking showed significance during the *Mid* period (Table 2). When there is a strong significant difference by day, it is driven by behavioral differences in the *Late* stage. There were substantial declines in eating behavior during the *Mid* period and drinking for the *Mid* and *Late* periods. These results could correlate to the significant mass loss in the *Late* period.

SPA tests that were performed prior to the start of the experiment all showed no presence of MG antibodies in all thirteen American goldfinches and the one house finch. All control birds did not exhibit the presence of MG antibodies throughout the duration of the experiment (Figure 12). Results from the ELISA-serum assay showed that 5 of the 7 infected birds seroconverted in fourteen days (Figure 12).

Table 1. Results of repeated measures ANOVA least squares effect tests examining the effects of infection with *Mycoplasma gallisepticum*, day relative to infection, and their interaction on physiological (mass and hemoglobin) and behavioral (perch, preen, cling, walk, jump, hop, eat, and drink) variables. *p*-values ≤ 0.05 are bolded and italicized.

Mass

	df	*F*	*p*
Treatment	1	0.02	0.88
Day	3	9.13	***<0.01***
Treatment x Day	3	7.92	***<0.01***

Hemoglobin

	df	*F*	*p*
Treatment	1	0.64	0.44
Day	3	0.78	0.54
Treatment x Day	3	1.31	0.32

Perch

	df	*F*	*p*
Treatment	1	6.20	***0.03***
Day	2	12.43	***<0.01***
Treatment x Day	2	13.32	***<0.01***

Preen

	df	*F*	*p*
Treatment	1	3.01	0.11
Day	2	5.67	***0.02***
Treatment x Day	2	1.96	0.19

Cling

	df	*F*	*p*
Treatment	1	0.31	0.59
Day	2	0.17	0.85
Treatment x Day	2	1.60	0.25

Walk

	df	*F*	*p*
Treatment	1	2.00	0.19
Day	2	3.56	0.07
Treatment x Day	2	0.69	0.52

Jump

	df	*F*	*p*
Treatment	1	0.95	0.36
Day	2	2.09	0.19
Treatment x Day	2	4.88	***0.05***

Hop

	df	*F*	*p*
Treatment	1	1.74	0.22
Day	2	0.72	0.53
Treatment x Day	2	1.50	0.30

Eat

	df	*F*	*p*
Treatment	1	0.01	0.93
Day	2	3.19	0.09
Treatment x Day	2	0.51	0.62

Drink

	df	*F*	*p*
Treatment	1	0.77	0.40
Day	2	2.15	0.17
Treatment x Day	2	3.15	0.07

Table 2. Results of repeated measures ANOVA least squares *post hoc* effect slices examining the interaction effects of infection and day relative to infection on host physiology (mass) and behavior (perch, preen, cling, walk, jump, hop, eat, and drink) variables. *p*-values ≤ 0.05 are bolded and italicized. For mass and hemoglobin, *Pre* refers to four days before the start of the experiment. *Early* refers to two days after inoculation. *Mid* refers to six days after inoculation. *Late* refers to thirteen days after inoculation. For behaviors, *Pre* refers to one day before the start of the experiment. *Mid* refers to five days after inoculation. *Late* refers to twelve days after inoculation.

Mass

	df	*F*	*p*
Pre	1	1.24	0.29
Early	1	1.47	0.25
Mid	1	2.31	0.16
Late	1	4.84	***0.05***

Hemoglobin

	df	*F*	*p*
Pre	1	2.59	0.13
Early	1	0.10	0.76
Mid	1	0.01	0.93
Late	1	0.02	0.88

Perch

	df	*F*	*p*
Pre	1	2.10	0.18
Mid	1	1.92	0.19
Late	1	98.11	***<0.01***

Preen

	df	*F*	*p*
Pre	1	0.55	0.47
Mid	1	2.69	0.13
Late	1	6.05	***0.03***

Cling

	df	*F*	*p*
Pre	1	0.00	0.98
Mid	1	0.17	0.69
Late	1	6.06	***0.03***

Walk

	df	*F*	*p*
Pre	1	1.04	0.33
Mid	1	0.17	0.68
Late	1	1.38	0.27

Jump

	df	*F*	*p*
Pre	1	2.61	0.14
Mid	1	7.44	***0.02***
Late	1	9.88	<***0.01***

Hop

	df	*F*	*p*
Pre	1	0.67	0.45
Mid	1	11.82	<***0.01***
Late	1	10.98	<***0.01***

Eat

	df	*F*	*p*
Pre	1	0.23	0.64
Mid	1	8.71	***0.01***
Late	1	3.89	0.07

Drink

	df	*F*	*p*
Pre	1	0.76	0.40
Mid	1	4.74	***0.05***
Late	1	1.59	***0.05***

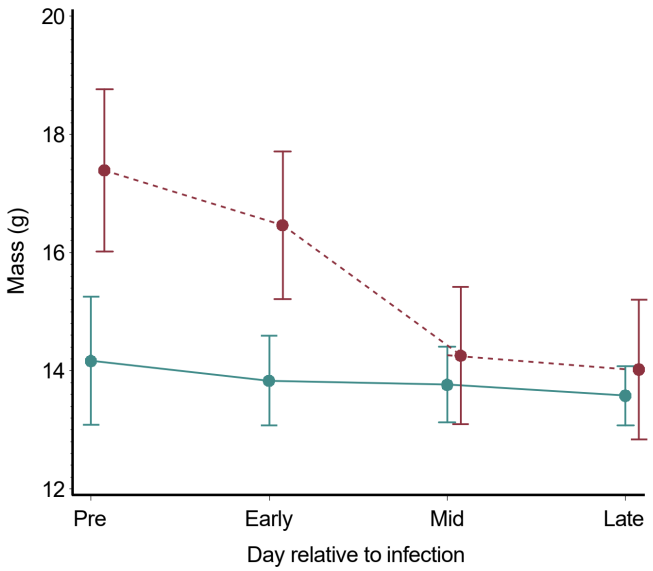

Figure 1. American goldfinch mass over the course of the study. Red filled dots represent infected birds. Green filled dots represent control birds. The bars above and below the dots are +/- standard error bars. *Pre* refers to four days before the start of the experiment. *Early* refers to two days after inoculation. *Mid* refers to six days after inoculation. *Late* refers to thirteen days after inoculation.

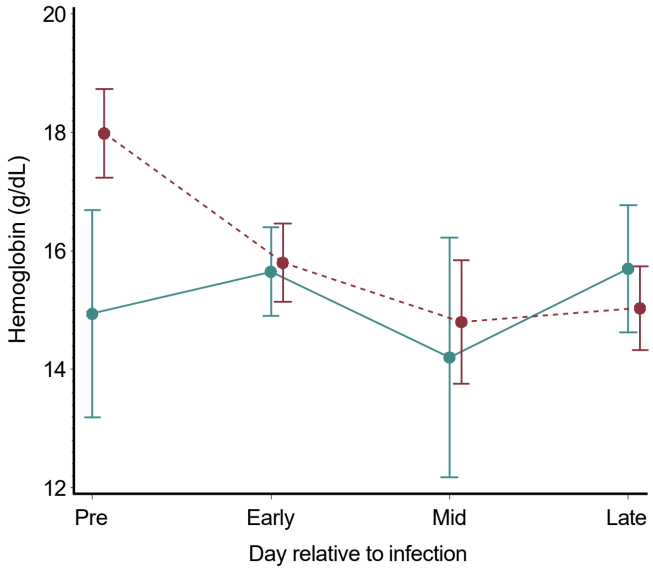

Figure 2. American goldfinch hemoglobin over the course of the study. Red filled dots represent infected birds. Green filled dots represent control birds. The bars above and below the dots are +/- standard error bars. *Pre* refers to four days before the start of the experiment. *Early* refers to two days after inoculation. *Mid* refers to six days after inoculation. *Late* refers to thirteen days after inoculation.

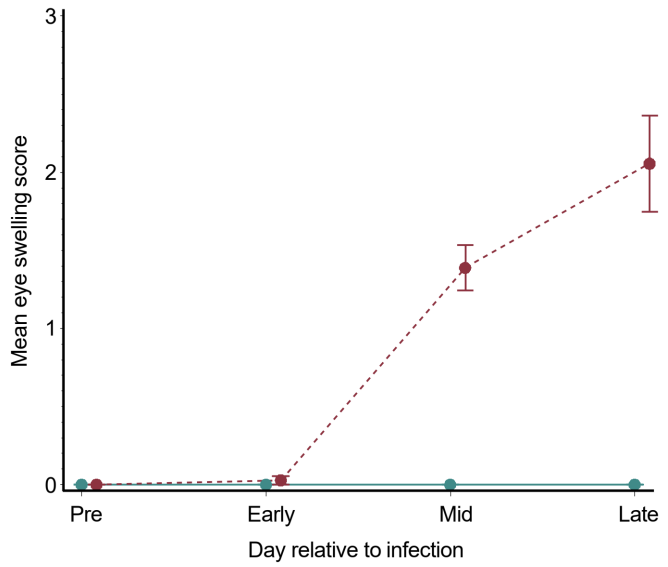

Figure 3. American goldfinch eye swelling over the course of the study. Red filled dots represent infected birds. Green filled dots represent control birds. The bars above and below the dots are +/- standard error bars. *Pre* refers to four days before the start of the experiment. *Early* refers to two days after inoculation. *Mid* refers to six days after inoculation. *Late* refers to thirteen days after inoculation.

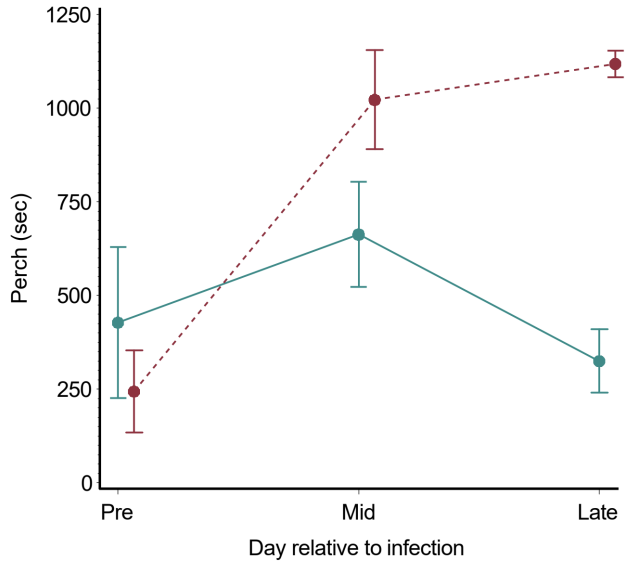

Figure 4. American goldfinch perching behavior over the course of the study. Red filled dots represent infected birds. Green filled dots represent control birds. The bars above and below the dots are +/- standard error bars. *Pre* refers to one day before the start of the experiment. *Mid* refers to five days after inoculation. *Late* refers to twelve days after inoculation.

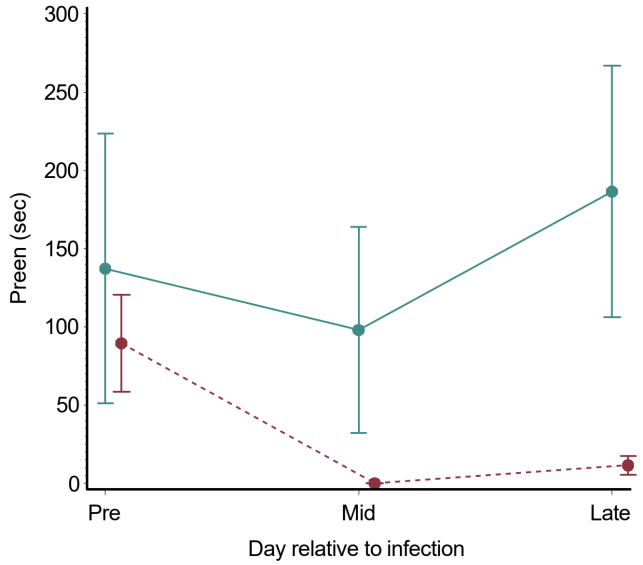

Figure 5. American goldfinch preening behavior over the course of the study. Red filled dots represent infected birds. Green filled dots represent control birds. The bars above and below the dots are +/- standard error bars. *Pre* refers to one day before the start of the experiment. *Mid* refers to five days after inoculation. *Late* refers to twelve days after inoculation.

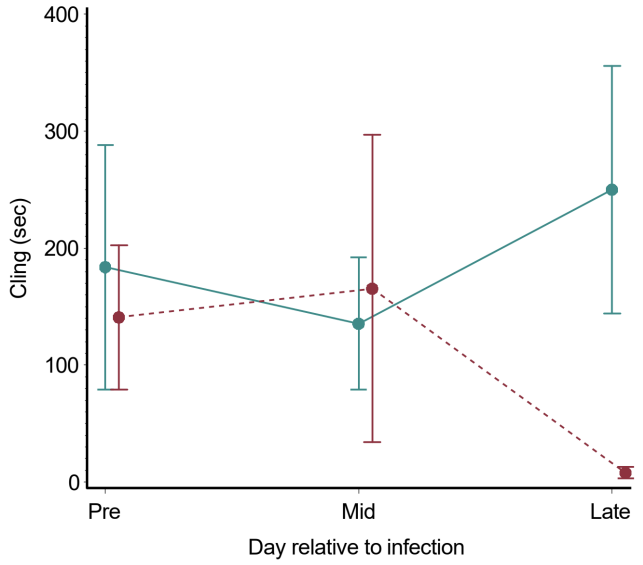

Figure 6. American goldfinch clinging behavior over the course of the study. Red filled dots represent infected birds. Green filled dots represent control birds. The bars above and below the dots are +/- standard error bars. *Pre* refers to one day before the start of the experiment. *Mid* refers to five days after inoculation. *Late* refers to twelve days after inoculation.

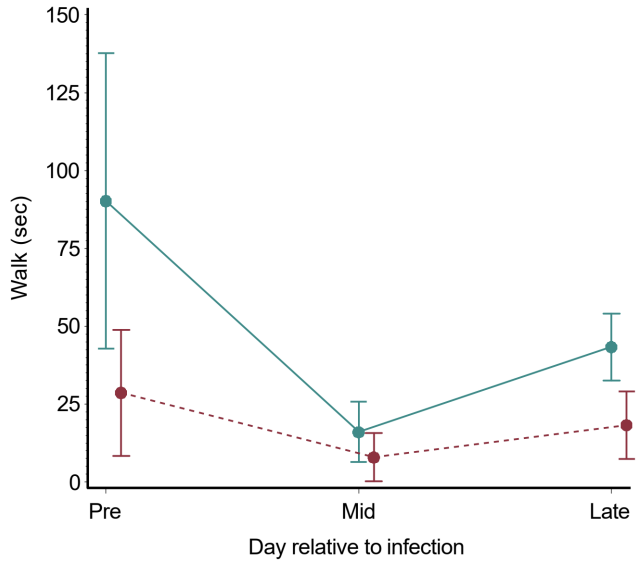

Figure 7. American goldfinch walking behavior over the course of the study. Red filled dots represent infected birds. Green filled dots represent control birds. The bars above and below the dots are +/- standard error bars. *Pre* refers to one day before the start of the experiment. *Mid* refers to five days after inoculation. *Late* refers to twelve days after inoculation.

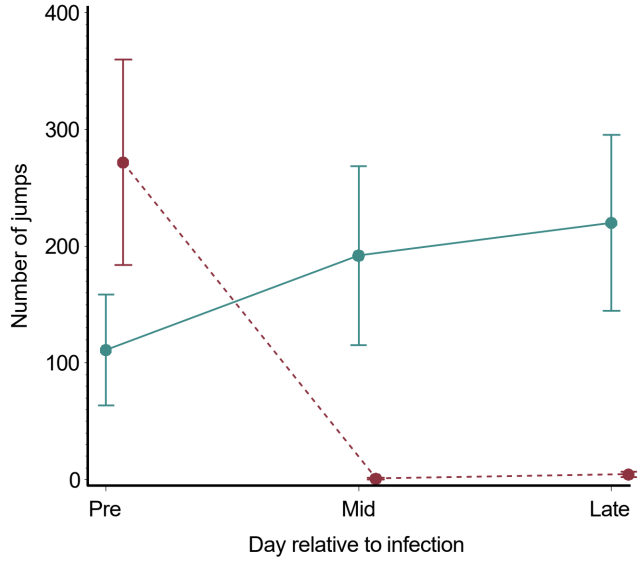

Figure 8. American goldfinch jumping behavior over the course of the study. Red filled dots represent infected birds. Green filled dots represent control birds. The bars above and below the dots are +/- standard error bars. *Pre* refers to one day before the start of the experiment. *Mid* refers to five days after inoculation. *Late* refers to twelve days after inoculation.

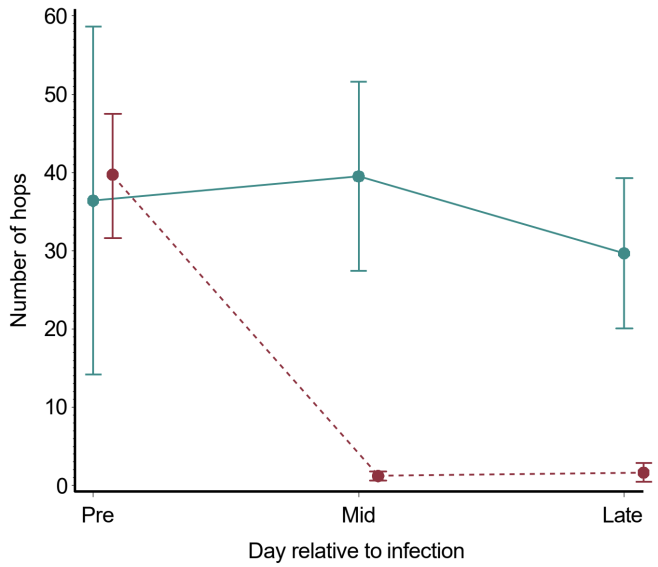

Figure 9. American goldfinch hopping behavior over the course of the study. Red filled dots represent infected birds. Green filled dots represent control birds. The bars above and below the dots are +/- standard error bars. *Pre* refers to one day before the start of the experiment. *Mid* refers to five days after inoculation. *Late* refers to twelve days after inoculation.

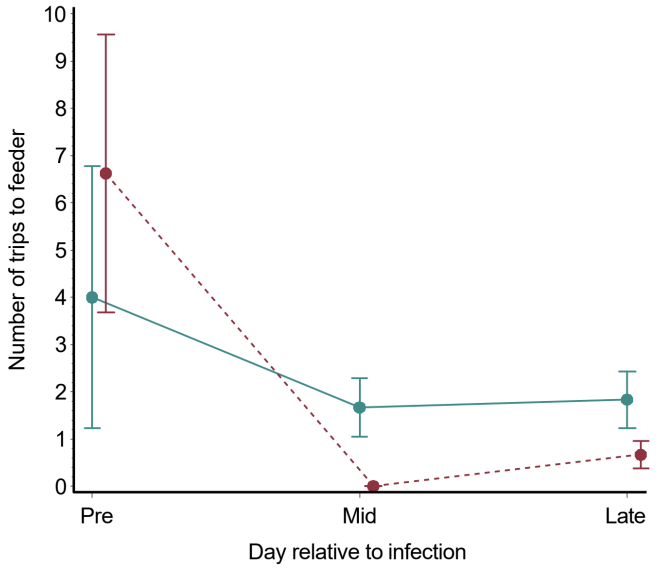

Figure 10. American goldfinch eating behavior over the course of the study. Red filled dots represent infected birds. Green filled dots represent control birds. The bars above and below the dots are +/- standard error bars. *Pre* refers to one day before the start of the experiment. *Mid* refers to five days after inoculation. *Late* refers to twelve days after inoculation.

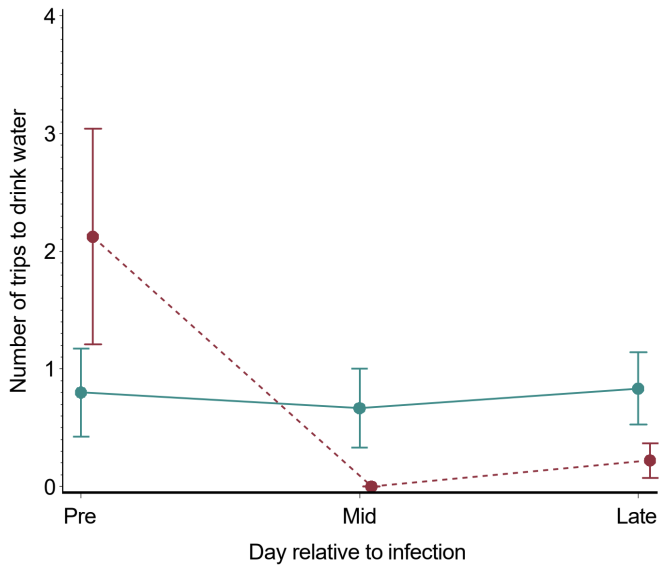

Figure 11. American goldfinch drinking behavior over the course of the study. Red filled dots represent infected birds. Green filled dots represent control birds. The bars above and below the dots are +/- standard error bars. *Pre* refers to one day before the start of the experiment. *Mid* refers to five days after inoculation. *Late* refers to twelve days after inoculation.

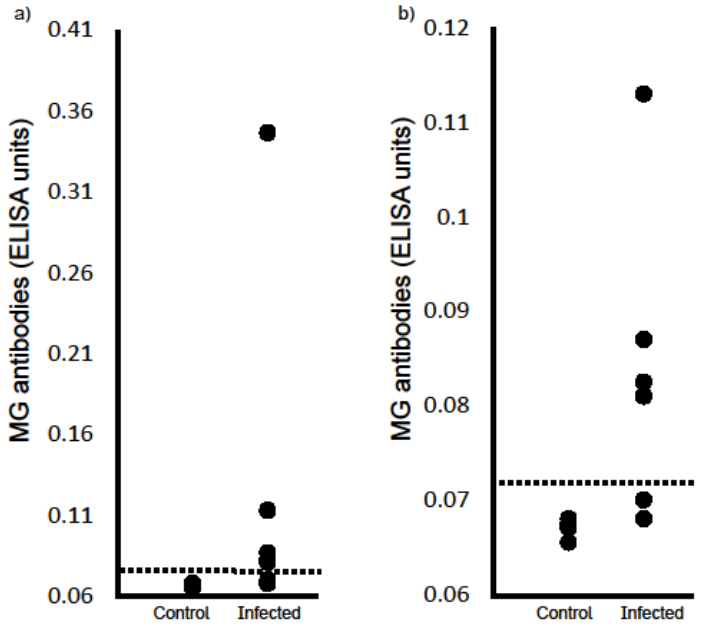

Figure 12. ELISA assay results showing relative amount of *Mycoplasma gallisepticum* antibodies in American goldfinches that were and were not infected for 14 days. The dotted line shows the break point in antibody levels between negative and positive. a) Relative antibodies for all birds included in the study. b) Relative antibodies for all but one bird included in the study to show the break in the data between negative and positive birds more clearly.

DISCUSSION

For this study, we examined the effect of MG infection on American goldfinch behavior. We present preliminary data that supports our hypothesis that the presence of infection and antibody development in the American goldfinch changes their behavior. MG infection does reduce movement in American goldfinches. Our results showed a significant difference in perching, preening, and jumping behaviors. The *Late* stage of the experiment exhibited the most significant decrease in behavior, as seen in jumping, hopping, drinking, preening, and clinging. Stationary behavior of perching showed a significant increase during the *Late* stage. We had defined perching as the behavior where the bird sits and does not perform any other activity, and it was the only behavior that significantly increased throughout the experiment. Because of the lack of other activities that we measured, the birds spent most of its time engaging in stationary behavior by perching on its branch or the food or water dish. All other significant active behaviors decreased.

Birds declined in mass throughout the experiment, with the most significant drop between the *Early* and *Mid* periods (Figure 1). The significant decrease in mass loss was a direct result of MG infection. Fatigue from infection could contribute to decreased physical activity, directly resulting from sickness behavior. Infection may cause inappetence and a reduction in feeding behaviors (Bonneaud et al., 2003; Adelman et al., 2017; Moyers et al., 2015; Bouwman and Hawley 2010; Lopes et al., 2021). Despite the easy accessibility to a food and water source, infected birds were not taking as much food and water as healthy birds. Therefore, they cannot

get the energy they need from a food source to be processed. This could directly contribute to its mass loss. Future studies could measure food intake and preferences in infected and uninfected birds. Because of American goldfinches' habit of picking at and throwing their food, food intake could be measured by surrounding each cage with a net and incorporating its weight in the grams of seed given and eaten daily.

We found no signs of anemia during the *Late* stage of MG infection. The hemoglobin of each bird also stayed relatively stable throughout the experiment. According to Samanta and Bandyopadhyay 2017, American goldfinches were observed to be carrying MG for an extended period without presenting any clinical signs. In contrast, Eastern bluebirds infected with the same strain of MG cultured from a house finch had shown anemia through low hemoglobin levels and significant splenomegaly (Balenger, unpubl. data).

We measured the level of conjunctivitis according to the severity of physical eye swelling. All controls birds showed no eye swelling. Mean eye swelling in MG-infected birds significantly increased over the experiment, especially between the *Early* to *Mid* period (Figure 3). The most severe conjunctivitis was present during the *Late* period (Figure 3). The eye swelling can hinder the American goldfinch's ability to see clearly (Dhondt et al., 2005). Previous studies have looked at the effects of conjunctivitis rendering the birds inactive during infection (Kollias et al., 2004; Dhondt et al., 2005). Furthermore, this can contribute to mass loss since the birds were not often traveling to their food and water dishes, as we observed in their behavior.

ELISA results showed that only 5 of the 7 infected birds seroconverted in fourteen days. This indicates more individual variation in how the American goldfinches respond

immunologically than house finches. American goldfinches have a lower MG DNA detected when infected than house finches (Dhondt et al., 2014). There is a low incidence in the detection of MG exposure compared to different species of the Fringillidae family (Dhondt et al., 2014). MG in American goldfinches may not be as common as house finches because these species are from a different genus (Dhondt et al., 2008).

American goldfinches and house finches belong to the same family of Fringillidae (Dhondt et al., 2014). Therefore, American goldfinches are more likely to be affected by the house finch-associated strain of MG (Allen et al., 2018). Dhondt et al. (2017) examined MG infection and reinfection rates in house finches. At their first exposure to MG, all house finches exhibited characteristics of an MG infection and symptoms of conjunctivitis. Reinfection with less virulent strains of MG developed an antibody response that allowed them to fight off the pathogen at a faster rate (Dhondt et al., 2017). However, more virulent strains of MG were harder to fight off, which allows the pathogen to prime house finches for further transmission (Dhondt et al., 2017). We used the house finch as our positive control because the house finches always have a strong antibody response to MG.

In conclusion, American goldfinches exhibited sickness behavior, as demonstrated by increased inactivity and mass loss during the *Late* stage of the experiment. We also saw an increase in conjunctivitis symptoms that corresponds to an increase in sickness behaviors as a result of MG infection. American goldfinches experienced more individual variation to MG compared to house finches (Farmer et al., 2002; Farmer et al., 2005). American goldfinches are at the greatest risk of mortality after the two week period for predation, dehydration, and starvation. The reduction of active behavioral responses supported our hypothesis. However, the

individual birds were not seen overcoming the infection and increasing their activity by the end of the experiment. This suggests the goldfinches could not fight off the infection as quickly. Our study would require a more thorough investigation into energy allocation during infection. Their immune response could be further studied to understand why hemoglobin did not differ and if it affected their survival negatively. Infection typically lowers hemoglobin levels and causes anemia (John 1994). In future work, we suggest using larger sample sizes and expanding the experimental timeline over a month. Behaviors, such as eating, drinking, jumping, and hopping, could also be measured as time in seconds instead of tally counts. The amount of eating and drinking can be quantified by measuring the mass of food and the volume of water consumed. Future studies can also consider the possibility of energy allocation and antibody development being subjected to individual variation. Our study demonstrates that American goldfinches also exhibit sickness behavior to MG infection similar in severity to house finches. Ultimately, this study can expand our perception of American goldfinch sickness behaviors and their role in the birds' survival rates during an MG infection.

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