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Authors: Sydenstricker, Keila V., Dhondt, André A., Ley, David H., and Kollias, George V.

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## RE-EXPOSURE OF CAPTIVE HOUSE FINCHES THAT RECOVERED FROM *MYCOPLASMA GALLISEPTICUM* INFECTION

Keila V. Sydenstricker,<sup>1</sup> André A. Dhondt,<sup>2,4</sup> David H. Ley,<sup>3</sup> and George V. Kollias<sup>1</sup>

<sup>1</sup> Wildlife Health Laboratory, College of Veterinary Medicine, Cornell University, Ithaca, New York 14853-6401, USA

<sup>2</sup> Laboratory of Ornithology, Cornell University, Ithaca, New York 14850, USA

<sup>3</sup> Population Health and Pathobiology, College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina 27606, USA

<sup>4</sup> Corresponding author (email: aad4@cornell.edu)

**ABSTRACT:** Fourteen house finches were reinoculated (re-exposed) with 0.05 ml ( $3.24 \times 10^5$  colony forming units/ml) of *Mycoplasma gallisepticum* (MG) in the conjunctival sac of each eye. All birds used in this reinoculation study had recovered from previous infection between 27 and 83 days after inoculation. Recovery was based on the absence of clinical signs of conjunctivitis and/or the inability to detect MG in conjunctival or choanal samples. Birds were maintained in individual cages under controlled environmental conditions at temperature 21–24 C, relative humidity 70%, and a light cycle adjusted to ambient values. They were divided into three groups, (A, B, and C). Five birds each were reinoculated 219 days (7.3 mo, group A) and 314 days (10.47 mo, group B) after the original infection. The final group of four birds was reinoculated at 425 days after experimental infection (14.17 mo, group C). Although the birds were randomly assigned to the three groups, the duration of the disease state (number of days until clinical signs last observed) during initial infection differed: group A mean =  $37.0 \pm SE 4.549$ , group B mean =  $63.6 \pm SE 6.306$ , group C mean =  $42.75 \pm SE 2.750$ ; analysis of variance  $F_{2,11} = 8.17$ ,  $P = 0.007$ . Within 24 hr after reinoculation six of the 14 experimental birds had developed some clinical signs of MG-induced conjunctivitis. At 3 days after reinoculation, 12 of the 14 birds had unilateral or bilateral conjunctivitis. The duration of clinical signs in the reinoculated individuals was significantly shorter than with their previous infection. These results suggest that the birds were able to mount a rapid and strong immune response following re-exposure. However, they were susceptible to reinfection and developed disease, suggesting that reinfection or perhaps even recurrence of infection and disease could occur in the free-ranging population. This may represent an important component in the epidemiology of this disease in house finches.

**Key words:** *Carpodacus mexicanus*, conjunctivitis, Eastern house finch, *Mycoplasma gallisepticum*, avian disease.

### INTRODUCTION

In 1994, *Mycoplasma gallisepticum* (MG) emerged as the cause of a major epidemic of conjunctivitis in house finches (*Carpodacus mexicanus*) in the eastern part of their range (Ley et al., 1996; Ley et al., 1997; Fisher et al., 1997; Dhondt et al., 1998). One of the more remarkable aspects of the dynamics of this disease is its seasonality. Disease prevalence is lowest in April–June, corresponding to the breeding season, but increases rapidly during summer, reaching a peak late fall. Prevalence is lowest in midwinter, but this is followed by a second increase in late winter (Dhondt et al., 1998; Altizer et al., 2004b). Increasing prevalence following the breeding season most likely relates to suscepti-

ble juveniles entering the population. The midwinter decline in prevalence is hypothesized to be caused by a herd immunity effect (John and Samuel, 2000); a sufficient proportion of the population has become immune to MG. It is also hypothesized that individuals recovering from an infection retain sufficient immunity, so that on re-exposure, they exhibit little to no signs of disease. It is possible that the late-winter peak could be related to a gradual loss of immunity that increases the number of susceptible birds. On the other hand, the retention of immune status or partial immunity for a year or more is consistent with our observation that conjunctivitis is observed less frequently in adults than in juvenile house finches (unpubl. observations).

In an earlier study, we experimentally infected house finches with MG and a high proportion of the birds (85%) survived and fully recovered from the disease (Kollias et al., 2004). Similarly, our field studies show that although diseased wild house finches suffer a higher mortality rate than nondiseased individuals, some birds do survive and recover (Faustino et al., 2004). In order to determine, under controlled conditions, how previously infected house finches that developed and recovered from conjunctivitis respond to a second exposure to the same MG strain, we reinoculated recovered house finches at approximately 7, 10, and 14 mo following the first experimental infection. The first infection caused a high morbidity (20/20) and low mortality (1/20); although most birds fully recovered, five became chronically infected (Kollias et al., 2004).

The purposes of this study were to determine whether house finches that had recovered from an experimental MG infection would be protected during a subsequent MG challenge and to characterize the duration of infection and duration and severity of the clinical response associated with this postinfection challenge.

## MATERIALS AND METHODS

### House finch capture sites

The house finches used in this experiment were trapped using mist nets or Potter traps in Tompkins County, New York, USA (42°51'N, 76°34'W) between June and October 2001 under permits from New York State Department of Environmental Conservation (Albany, New York, USA: No. LCP 99-039) and a federal collecting permit (PRT 802829).

### Animal care and use

All procedures and protocols were reviewed and approved by the Cornell University Institutional Animal Care and Use Committee (protocol 00-90). All birds were determined to be first-year individuals based on plumage characteristics and degree of skull pneumatization (Pyle, 1997). After capture, birds were housed individually in cages and allowed to acclimatize for a minimum of 4 wk. Birds were initially infected with MG on 13 November 2001 (sixth *in vitro* broth passage from the original MG

house finch isolate, ADRL 7994-1; Ley et al., 1996) by instillation of droplets into the conjunctival sac of each eye with 0.05 ml of the inoculum ( $3.24 \times 10^5$  colony forming units/ml). The 14 birds used in the reinoculation experiment were considered to be recovered between 27 to 83 days after inoculation, when they no longer had clinical signs of conjunctivitis and/or detectable MG in conjunctival or choanal samples.

Each bird was held singly in wire bar cages measuring 45 cm high  $\times$  45 cm wide  $\times$  75 cm long. Metal barriers between cages prevented possible cross contamination with MG between birds in different cages. Cages were arranged on the walls of a BL-2 biosafety isolation unit (Poultry Virus Isolation Facility) at the College of Veterinary Medicine, Cornell University. In each cage arrangement of perches, water and food bowls were identical. Water and a pelleted diet (Roudybush, Inc. Cameron Park, California, USA) were offered *ad libitum*. The birds were maintained under controlled environmental conditions (temperatures 21–24 C, relative humidity 70%, and light cycle adjusted to ambient values).

### Sampling, polymerase chain reaction, serology, and microbiology

Five birds were reinoculated on 20 June 2002 (group A; 219 days or 7.3 mo following the first inoculation), and five more were reinoculated on 23 September 2002 (group B; 314 days or 10.47 mo following the first inoculation). Four birds were inoculated on 12 January 2003 (group C; 425 days or 14.17 mo following the first inoculation).

Although the birds were assigned at random to the three groups for reinfection, the responses of the birds in the three groups to the first infection differed significantly in duration of disease state (number of days until clinical signs last observed: group A,  $37.0 \pm \text{SE } 4.549$ ; group B,  $63.6 \pm \text{SE } 6.306$ ; group C,  $42.75 \pm \text{SE } 2.750$ ; one-way analysis of variance (ANOVA)  $F_{2,11} = 8.17$ ,  $P = 0.007$ ). The response of the birds assigned to the three groups, however, did not differ significantly in severity of disease as measured by maximal eye score (one-way ANOVA  $F_{2,11} = 1.90$ ,  $P = 0.20$ ), nor in duration of pathogen presence in conjunctival and choanal samples detected by polymerase chain reaction (PCR) (Kruskall-Wallis ANOVA,  $H = 4.994$ ,  $P = 0.082$ ). In each group, PCR and serology were included for two newly-captured juvenile birds, proven to be MG-free by eye score (described below). One was used as a positive control and one as a negative control. The negative control bird was inoculated with

the same volume of nonpreservative sterile physiologic saline solution. Since the three negative controls did not develop physical signs or seroconvert, they are not described in the results section. The other newly captured bird in each group was a positive control bird that was inoculated with MG for the first time using the same methodology as for the birds that were reinoculated. We compared the response of the positive control birds with the individuals that were reinoculated and with the response of these same birds when they were initially infected in November 2001.

Before reinoculation, all birds in the study were tested for MG by PCR testing of conjunctival and choanal swabs (calcium alginate fiber-tipped ultrafine aluminum applicator swab, catalog number 14-959-78, Fisher Scientific, Pittsburgh, Pennsylvania, USA). Swabs were placed in Frey's medium with 15% swine serum (Kleven, 1998) and were submitted to North Carolina State University (NCSU) for verification of MG presence by PCR (Lauerman, 1998), following incubation at 37 C for 1 wk. Blood was collected from the left wing vein into two to three lithium-heparinized microcapillary tubes for use in a rapid plate agglutination assay (RPA) (Kleven, 1998) for the detection of MG antibody.

During the first 10 days after inoculation, birds were observed daily to determine development and degree of conjunctivitis. Physical signs of MG infection were quantified by scoring the character and severity of the inflammatory response in each eye throughout the experiment. An eye scoring system previously described (Kollias et al., 2004) was modified by limiting the maximum score to three. A score of zero represented a bird with no clinical signs; a bird with a score of one had pink (not red) conjunctival discoloration and slight periorbital edema; a score of three indicated that a bird exhibited epiphora and feather matting, feather loss around the periorbital ring, moderate to severe conjunctival edema, and at least some chemosis and or rhinitis.

Following an initial 10-day period, the eye scores of all the birds were recorded twice a week for an additional 60 days (59 days in group C). At days 1, 5, 10 or 11, 40 or 42, and 59 or 60, after inoculation samples from both conjunctival sacs and choanal cleft were collected for MG PCR analyses as previously described. Blood samples for RPA testing were collected before inoculation and on days 5, 10, 22, 40, and 59 or 60 after inoculation.

#### Data analysis

We used the following response variables in the analyses reported below: *Eye score* = eye

score per eye averaged over both eyes in an individual on a given day. *Duration of disease state* = number of days until no clinical signs were observed. If on successive dates eye scores were zero and nonzero, indicating a variable and mild inflammatory reaction, we used the last date with score of zero. *Duration of pathogen presence* = the length of time MG was detectable by PCR from the conjunctival sac and choanal samples. For comparisons with results of the initial infection study, we based this on conjunctival swab results only; during initial infection the choanal cleft was sampled only three times. A bird was considered negative for MG if PCR results were negative for at least three consecutive sampling periods.

Response variables were compared among groups A, B, C; between all reinoculated birds, and the control birds; between seropositive and seronegative birds at the time of reinoculation, and between sexes. For each individual, we compared response during the initial and second experimental infection.

Parametric one-way ANOVA was used when variances were not significantly different, and Kruskal-Wallis ANOVA when variances differed significantly according to Bartlett's test of equal variance. When only two samples were compared, a Student's *t*-test (correcting for unequal variances if needed) was used (Statistix 7.0, Analytical Software, Tallahassee, Florida, USA). Paired comparisons were carried out using either a paired *t*-test or a Wilcoxon matched-pairs signed ranks test (Siegel, 1956).

To compare changes in eye score over time, repeated measure mixed models (PROC MIXED in SAS, 2001) were used. The change in eye score, in response to reinoculation during the initial 5 days after inoculation, was tested to determine whether they differed significantly among groups, between sexes, and with serologic status. A 5-day period was chosen because eye scores of reinoculated birds increased linearly during that period (Fig. 1). These three variables were included as categorical variables, and day after inoculation as a continuous variable. The initial model also contained the two-way interaction terms between day and each main effect because a significant interaction term would show that the increase in eye score after inoculation varied with the main effect. The "compound symmetry" option for the covariance structure was selected to allow for individual differences in response. The best model was selected by sequentially removing the nonsignificant (>0.10) interaction terms followed by removal of the nonsignificant main effects that were not included in a significant interaction term.

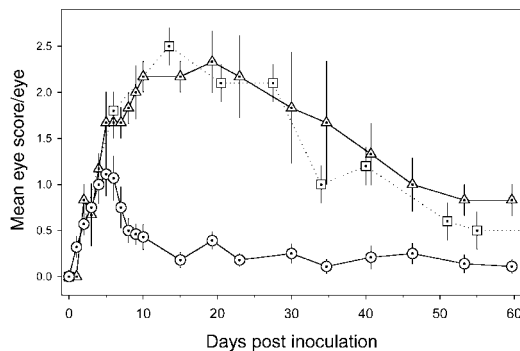


FIGURE 1. Change in clinical signs of conjunctivitis in house finches as marked by mean eye score over the course of the experiment (mean per eye  $\pm$  SE). Squares represent eye scores of 14 individuals when initially inoculated with *Mycoplasma gallisepticum* in 2001. Open circles are for the same 14 individuals when reinoculated 7, 10, and 14 mo after initial inoculation. Open triangles represent eye scores of three naive positive control individuals.

## RESULTS

### Eye score

Within 24 hr after reinoculation, six of the 14 experimental birds had developed some clinical signs of conjunctivitis. Three days after reinoculation, 12 of the 14 birds had unilateral or bilateral conjunctivitis. One of the remaining birds had bilateral conjunctivitis on day 5 after reinoculation only, whereas the other did not develop clinical signs. Mean eye score per eye gradually increased and reached a maximum of  $1.11 \pm \text{SE } 0.235$  on day 5 after reinoculation, and all birds gradually recovered (Fig. 1). On day 15 after reinoculation, 10 birds exhibited no signs of conjunctivitis (eye score of 0). In one bird, conjunctivitis was still present when the experiment ended on day 60 (Fig. 1). The response to reinoculation ranged from no detectable disease to birds exhibiting conjunctivitis beginning within 24 hr after reinoculation and persisting up to 60 days after reinoculation.

The average eye score of the reinoculated birds began to deviate from that of the positive control birds at day 5. On day 6 there was a tendency for the eye score between the control and reinoculated birds to

diverge ( $t_{11,3}=2.04$ ,  $P=0.065$ ), and the eye scores of the reinoculated birds were significantly lower from day 7 after reinoculation onward ( $t_{10,3}=3.31$ ,  $P=0.008$ ), indicating an onset of recovery. Most birds recovered within 2 wk. All birds, except one in group C, had fully recovered (eye score = 0) by day 40 after reinoculation.

The duration of clinical signs was significantly shorter in the 14 reinoculated birds than in the three positive control birds (treated as a group) (two-tailed Mann-Whitney  $U$ -test,  $U=2.3$ ,  $P=0.019$ ). Similarly, the duration of clinical signs in the reinoculated individuals was significantly shorter than observed in their initial infection: the average duration of clinical signs during the first infection was  $48.14 \pm 4.214$  days as compared with only  $14.57 \pm 4.740$  days after reinoculation (Wilcoxon matched-pairs signed ranks test  $n=14$ ,  $T=0$ ,  $P=0.001$ ).

All positive controls developed the first signs of conjunctivitis 2 days after inoculation, and continued to exhibit clinical signs until the end of the experiment on day 60. The maximum eye score was observed at an average of  $17.3 (\pm \text{SE } 5.607)$  days after inoculation. These results are consistent with results from the 14 birds during their initial inoculation (Fig. 1), demonstrating that previously unexposed birds responded in a similar way in the 2001 and 2002 experimental studies.

A mixed model ANOVA did not show an effect of group, antibody status, or sex on the duration of the eye infection (all  $P>0.2$ )

### Rapid plate agglutination (RPA)

Seven of the reinoculated birds were antibody negative prior to reinoculation; seven were antibody positive. The RPA-positive birds remained positive throughout the experiment, and all RPA-negative birds seroconverted. Three birds seroconverted by day 5 after reinoculation and remained positive until the end of the experiment at day 60. The remaining four birds seroconverted by day 10 after rein-



TABLE 1. Results from a repeated-measures mixed model testing for effects of group, sex, and antibody status on eye score during days 1–5 after inoculation. We report only the significant effects.

Effect	Numerator df	Denominator df	F	P
Day	1	54	30.36	<0.0001
Antibody status	1	12	0.59	0.46
Day×Antibody	1	54	6.10	0.017

Effect	Solutions				
	Estimate	SE	df	t	P
Intercept	0.029	0.447	12	0.06	0.95
Day	0.600	0.106	54	5.64	<0.0001
Antibody negative	0.486	0.631	12	0.77	0.46
Day×Antibody negative	-0.371	0.150	54	-2.47	0.017

oculation. Two of these tested RPA negative by day 40, one tested negative on day 60, and one remained seropositive. Two positive control birds seroconverted by day 5 and remained positive. One positive control bird seroconverted by day 10 but became seronegative beginning at day 22 after reinoculation.

Because the three groups contained roughly equal numbers of antibody positive and antibody negative birds, and a roughly equal sex ratio, we were able to carry out a single statistical analysis using PROC MIXED in SAS to determine

whether eye score changes during the initial 5 days after inoculation differed significantly among groups, between sexes, and with antibody status. Neither group effect nor sex effect was found although antibody status significantly influenced the initial response to reinoculation. The final model is given in Table 1, and the results are illustrated in Figure 2. We find significant effects of Day and of the interaction term Antibody status×Day. Birds that were RPA positive before reinoculation had a more severe response to reinoculation than RPA-negative birds.

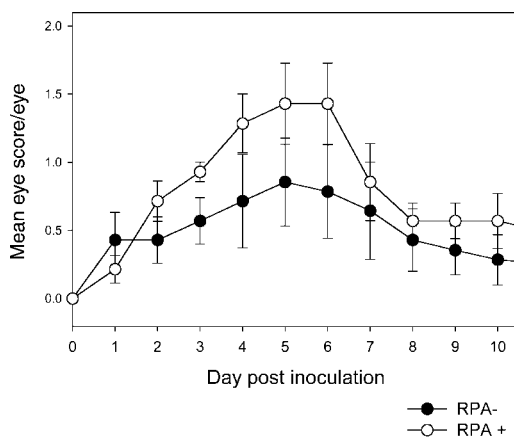


FIGURE 2. Mean eye score of house finches that were reinoculated 7, 10, and 14 mo after initial inoculation with *Mycoplasma gallisepticum* (MG). Open circles represent seven individuals in which MG antibodies were detected before reinoculation; filled circles are for seven individuals in which MG antibodies were absent.

#### Polymerase chain reaction (PCR)

One bird in group A did not develop clinical signs, remained MG PCR negative, but did seroconvert. In 12 of the 13 remaining birds, the conjunctival samples were MG PCR positive (PCR+) on day 1. The ability to detect MG decreased rapidly, however, with only two birds remaining PCR+ on day 11. *Mycoplasma gallisepticum* was detected in choanal samples at later sampling dates and less frequently than in conjunctival samples. On day 1 after reinoculation, choanal swabs from only six birds tested PCR positive, which is significantly less frequently than in conjunctival samples (two-tailed Fisher exact test  $P=0.046$ ). In three birds that were PCR+ from conjunctival samples, MG was not detected from the choanal samples. Thus,

only 10 of the 14 reinoculated birds tested PCR+ from choanal swabs.

*Mycoplasma gallisepticum* was detected from both conjunctival and choanal swabs from the three positive control birds. Conjunctival samples of the positive controls tested PCR positive for an average of  $37.3 \pm 14.05$  days, which is significantly longer than  $8.50 \pm 3.607$  observed for reinoculated birds (two-tailed Mann-Whitney *U*-test,  $U=2.4$ ,  $P=0.015$ ). During the first inoculation study, MG was also detected from both conjunctival and choanal swabs, and the mean duration for detecting MG by PCR was  $31.86 \pm 4.346$  days, which was significantly longer than occurred following reinoculation (Wilcoxon matched-pairs signed ranks test:  $n=13$ ,  $T=7$ ,  $P=0.008$ ). In positive controls, the duration infection, as detected by MG PCR, was the same when based on conjunctival or choanal samples.

We detected MG in choanal swabs significantly less often in group A birds (1/5) than group B birds (5/5) (two-tailed Fisher exact probability test  $P=0.048$ ). There was no difference between group B (5/5) and group C (4/4) birds.

#### DISCUSSION

Although there is extensive information available on MG infection and immunity in chickens and turkeys (Ley, 2003), little information is available for house finches. In recent studies, it was demonstrated that house finches develop some immunity after being exposed to MG (Roberts et al., 2001; Farmer et al., 2002; Ferguson et al., 2003; Kollias et al., 2004).

Our study demonstrates that house finches that recovered from MG infection exhibit partial immunity to MG infection when challenged 7–14 mo later. The incubation period following reinoculation was approximately 1 to 3 days, which was shorter than the response during initial inoculation (2–6 days) and from results reported by Farmer et al. (2002). On average, reinoculated birds and inoculation of previously unexposed birds exhibited the

same degree of response (eye score) during the initial 5 days after inoculation, but the eye scores in previously unexposed birds continued to increase and reached a maximum at 2–3 wk after inoculation. Reinoculated individuals began to recover on day 6, with most birds being fully recovered within 2 wk after reinoculation. Our results show that in birds that have recovered from previous MG infections, a protective immune response is not apparent until 4–5 days after reinoculation, at which point birds rapidly recover. Most birds (10/14) had an eye score of zero 15 days after reinoculation, and only 2/14 of the birds remained MG PCR positive in conjunctival swabs at that time.

Although there was no significant difference in the severity of clinical signs observed in reinoculated birds based on eye score, the three groups did differ in relation to detection by PCR. In the group that was reinoculated after 7 mo, fewer birds had MG identified from choanal swabs as compared with the birds that were reinoculated after 10 or 14 mo.

The observed response to reinoculation was extremely variable. One bird exhibited an eye score of zero and appeared to clear MG within 24 hr based on PCR. In contrast, one bird developed a chronic infection and conjunctivitis persisting for 60 days after reinoculation. Differences in severity of eye scores could not be linked to the interval between initial and re-exposure or to sex. However, in birds that were seropositive prior to reinoculation, clinical signs developed more rapidly and were more severe (higher eye score) when compared with the seronegative individuals (Fig. 2). In our study, there is no clear correlation between the presence of MG antibody and resistance to infection. Birds became infected and developed conjunctivitis even though they have antibody. Local or mucosal antibody production could be more important for resistance to MG since MG colonizes mucosal and serosal surfaces (Levisohn and Kleven, 2000) and may be important in house finch MG in-

fection. Chickens re-exposed to MG exhibited increased antibody titers to MG in tracheal washings with a decrease in the number of organisms and tracheal lesions (Yagihashi and Tajima, 1986), demonstrating that local immunity was present.

House finches reinoculated 7 to 14 mo after an initial infection were able to mount an immune response and clear infection much more rapidly than naive birds exposed to MG, a result that has also been observed in chickens re-exposed to MG. (Yagihashi and Tajima, 1986). Ferguson et al. (2003) demonstrated that turkeys infected with MG strain K5054 of lower virulence but very similar to the house finch strain developed some immunity and subsequently exhibited resistance when challenged with a virulent MG strain (R strain).

In summary, house finches reinoculated with MG developed milder disease than observed at initial exposure or when compared with a naive control group. Re-exposed house finches also exhibited clinical signs for a shorter period of time. Additionally, we hypothesize that because individuals were able to mount a rapid and effective immune response against MG, the effectiveness of MG detection by PCR testing of choanal swabs was reduced. Additionally, our results show that house finches exhibited partial immunity to challenge with MG more than 1 yr after recovery from a primary MG infection. This might explain why adult wild house finches show clinical signs less frequently than juveniles (Altizer et al., 2004a). Our results also show house finches to have acquired immunity lasting between 7 and 14 mo after an initial infection. This suggests that our hypothesis explaining the late-winter peak in disease prevalence should be rejected.

Because we have shown that house finches are susceptible to reinfection, this supports the hypothesis that reinfection, or even recurrence of infection, could occur in the free-ranging population, which

would be an important factor in the epidemiology of this disease.

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