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Response of house finches recovered from *Mycoplasma gallisepticum* to reinfection with a heterologous strain.

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Summary

After recovery house finches reinfected with the same *Mycoplasma gallisepticum*-strain remain partially resistant to reinfection for at least 14 months in that they recover from reinfection much more rapidly than *Mycoplasma gallisepticum*-naïve birds. To test the response of birds to reinfection with a heterologous strain we performed two experiments. In a first experiment we exposed birds to one of three strains that differed in virulence. After they had recovered all were reinfected with the most virulent strain available at the time of the experiment. In a second experiment we infected and later reinfected house finches with one of two *Mycoplasma gallisepticum* strains, whereby we switched the order of the strain used. In both experiments disease in birds reinfected with a more virulent strain caused more severe disease. Our data suggest that the observed increase in *Mycoplasma gallisepticum* virulence, once the disease has become endemic in free-ranging house finches is – in part – driven by increased resistance of recovered birds to strains of equal or lower virulence.

Keywords

virulence; emerging disease

Mycoplasmal conjunctivitis in house finches results from infection with the bacterium *Mycoplasma gallisepticum* (6, 18). This disease emerged in the early 1990s when *Mycoplasma gallisepticum*, a well-known pathogen of poultry, successfully colonized a songbird host, the house finch (13), resulting in an epidemic that spread across North America (2, 4). In house finches the disease is associated with severe conjunctivitis, sinusitis, and rhinitis whereas in poultry it causes respiratory disease characterized by tracheitis and airsacculitis (17). Although *Mycoplasma gallisepticum* causes severe and extended disease in house finches (16) a high proportion of infected birds recover (5).

Especially in late summer and fall, 50% or more of the individuals in a population can be infected (1, 2, 9, 12, 15). The disease has become endemic in many parts of the house finch range.

As the epidemic spread in space and time the pathogen evolved (13, 22) and once established in a region it increased in virulence (10, 11). Birds infected with strains of higher virulence carried a higher bacterial load in the conjunctiva, and developed more severe disease for longer periods (11), and therefore transmitted the infection more successfully (24).

As part of our study on *Mycoplasma gallisepticum* disease dynamics, we measured the response of house finches recovered from a first infection to a 2nd exposure with the pathogen. In our first such experiment, we used the same strain for the first and for the 2nd infection and found that recovered birds remained more resistant to reinfection up to at least 14 months after the first infection (21). We considered an individual to have recovered if we could not detect *Mycoplasma gallisepticum* from conjunctival swabs using qPCR (8) and eye lesions had been completely resolved. Recovered birds may not have cleared *Mycoplasma gallisepticum* completely (3). To determine if birds that had recovered from a first infection would respond differently to reinfection with a heterologous strain, we carried out further reinfection experiments to address the following two hypotheses. The first hypothesis posits that response to reinfection is asymmetrically driven by differences in virulence between the first and the second infection. It assumes that disease severity in the recovered birds upon reinfection will depend on the degree to which the second strain is more virulent than the first one: the larger the difference in virulence between two strains, the more severe the response to reinfection will be. If the second strain is of lesser virulence than the first, upon reinfection birds should develop no or very limited disease. The second hypothesis proposes that response to reinfection is modulated by the genetic difference between two strains, as found in experiments with avian influenza virus in black-headed gulls *Chroicocephalus ridibundus* (23): it assumes that birds are more resistant to a subsequent infection with the same strain than to reinfection with a heterologous strain regardless of its virulence; reciprocal treatments with heterologous strains should therefore have similar effects.

We performed two sets of experiments to begin to address these hypotheses. In Experiment A, we exposed house finches to one of three *Mycoplasma gallisepticum* strains that differed in virulence and waited until they had fully recovered. We then reinfected the birds with the most virulent strain available at the time of the experiment, i.e. NC2006. In Experiment B we performed reciprocal reinfection experiments with one of two heterologous strains: VA1994 - a strain of lower virulence, and NC2006 - one of higher virulence; we reversed the sequence in which birds were exposed to the two strains.

The first hypothesis would be supported if in Experiment A, disease caused by reinfection with the virulent NC2006 strain would decrease in severity with increasing order of strain virulence used in the first infection, as follows: (1) negative control (strongest disease response) > (2) CA2006 (low virulence) > (3) VA1994 (intermediate virulence) > (4) positive control NC2006 (high virulence; weakest response). Hypothesis 1 also predicts that

in Experiment B reinfection with VA1994 after recovery from an infection with NC2006 would cause mild disease only, while reinfection with NC2006 after recovery from an infection with VA1994 would cause severe disease.

The second hypothesis predicts that in experiment B, the response to a heterologous strain would be independent of the sequence in which the strains were used and should be approximately equal.

MATERIAL AND METHODS

Origin of birds - permits

Juvenile house finches were trapped in Ithaca, NY using mist nets in the fall of 2009. Trapping was performed under permits 22669 (USGS, Patuxent), and 99-039 (NY Department of Environmental Conservation). Birds were kept in quarantine and only juvenile house finches negative for evidence of previous exposure to *Mycoplasma gallisepticum* as determined by absence of eye lesions, no bacterial DNA detected by qPCR (8) and absence of antibodies (7, 20) were included. All procedures were approved by Cornell University IACUC protocol #2006-0094.

Bird housing

In the first part of Experiment A, groups of 12 birds were housed in spacious octagonal aviaries inside a large barn with artificial light, but no heating. Each aviary had a volume of 17.87 m³, and contained one large six-port tube feeder (refilled daily with Roudybush Maintenance) and a water bath, heated by a heating lamp in winter. The water bath was cleaned and refilled daily. Two artificial trees were placed in a corner, and several plastic perches were attached to the walls. Close to one of the perches, we also provided a ceramic heating lamp during winter. At about 1.80 m height, we hung five artificial wreaths that birds used for roosting. For experiments in which birds were kept individually we used cages that were 45 cm high × 45 cm wide × 75 cm long. The cages were separated with metal barriers to prevent possible mechanical cross-contamination of *Mycoplasma gallisepticum*. Each cage contained perches, food, and water ad libitum.

Mycoplasma gallisepticum strains used

We used following inocula: the low virulence strain CA2006 [2006.052-5 (4p) 1/13/2009 no dilution]; the index strain of intermediate virulence VA1994 [7994-1 (7p) 2/12/2009 dilution 1:7.4]; the high virulence strain NC2006 2006.080-5 (4p) 1/9/2009 dilution 1:100. Dilutions were made with Frey's medium so that all inocula had the same color-changing units (CCU) (11, 19).

Experiments

Experiment A – Reinoculation with NC2006, a virulent strain.

1st exposure: Six groups of 12 juvenile house finches, negative for previous exposure to *Mycoplasma gallisepticum*, were each placed in a large aviary. On 4 February 2010 two individuals of each group were chosen at random and inoculated in the conjunctival sac with 0.05 ml of inoculum, kept in a paper bag for 10 minutes to allow the inoculum to be

absorbed, and released back into the aviary. One of three *Mycoplasma gallisepticum* inocula listed above was used in two groups each. Control birds were inoculated in the conjunctiva with 0.05 ml of Frey's medium.

These birds were studied until 8 June 2010 (day 127 post infection (PI)). Using the method described in (19) eye lesion scores for each eye were recorded on 13 dates starting day 4 PI and then on day PI 11, 18, 25, 32, 39, 46, 58, 70, 85, 99, 113, and 127. In each eye a conjunctival swab to measure *Mycoplasma gallisepticum* load (number of copies of *mgc2* gene) using qPCR (8) was taken on the same dates. A small blood sample was taken to measure the level of antibodies using ELISA (7) on day PI 11, 25, 39, 58, 85, and 113. The detailed results of this experiment whose objective was to compare transmission rates between strains has been reported elsewhere (24).

2nd exposure—: From the experiment described above, we selected individuals for which we had evidence that they had been infected with *Mycoplasma gallisepticum* (seroconversion and/or bacteria were detected in a conjunctival swabs using qPCR). These birds were placed in individual cages. On 1 November 2010, after having confirmed that birds no longer carried *Mycoplasma gallisepticum* detectable by qPCR, they were inoculated in each eye with the more virulent NC2006 strain. We reinoculated the following numbers of birds: 8 that were originally infected with CA2006, 8 that were originally infected with VA1994, and 12 that were originally infected with NC2006. We also inoculated 12 control birds that had originally been sham-inoculated with Frey's medium and had not developed any signs of infection. We took conjunctival swabs for qPCR analyses on days 3, 7, 21, 28, 35 PI, and recorded eye score on days 3, 7, 14, 21, 28, 35, 43, 50, 64, 71, 79, 91 PI. To compare responses to treatments we summed the values of $\log(\text{mgc2} + 1)$ to day 35 PI as a measure of *Mycoplasma gallisepticum* load, and summed the eyescore (left + right) to day 91 PI across all days sampled for each individual. These sums integrate the response to infection across the duration of the experiment.

Experiment B. Reciprocal re-infection with heterologous strain.

- *VA1994 first, NC2006 second.* As part of Experiment A eight naïve birds were exposed to VA1994 on 1 February 2010 and reinoculated with NC2006 on 1 November 2010.
- *NC2006 first, VA1994 second.* Seven birds, held in individual cages, were inoculated on 1 November 2010 with NC2006 and reinoculated with VA1994 on 31 May 2011.
- Five control birds, naïve for *Mycoplasma gallisepticum*, were inoculated with VA1994 on 31 May 2011.

Only birds that had fully recovered from an earlier infection were included in these experiments. We recorded eye scores and took conjunctival swabs (to measure *Mycoplasma gallisepticum* load) on day 4 and 7 PI, and then weekly until day 35 PI. No conjunctival swab sample was taken on day 14 PI.

Statistical analyses

Analysis of variance followed by the post-hoc Scheffé test were used to compare variables in experiments with more than two treatments. We analyzed the results of Experiment B with a Mann-Whitney U-test. Calculations were performed with Statistix 10, Tallahassee, FL software.

RESULTS

Experiment A.

The response of birds reinfected with the more virulent NC2006 strain varied between treatments (Fig. 1; Table 1). All birds reinfected with a heterologous strain maintained a *Mycoplasma gallisepticum* infection and developed eye lesions, although its duration and severity varied between treatments as shown by One-Way-ANOVAs (bacterial load: $F_{3,36} = 76.24$, $P < 0.0001$; eye lesions: $F_{3,36} = 29.74$, $P < 0.0001$). The Scheffé post-hoc test ($\alpha = 0.05$) showed that the bacterial loads differed between all groups, while there was some overlap between groups regarding the eye lesions as indicated in Table 1.

Bacterial load was highest in the control birds, lower in birds previously exposed to CA2006, and lower still in birds recovered from a previous VA1994 infection. In birds that were exposed twice to NC2006 no bacteria were detected the 2nd time, although in one bird mild eye lesions were observed on day 3 PI, and although in 9 of the 12 birds antibodies were detected.

Experiment B

Of the 7 birds that had recovered from a first exposure to NC2006 and that were reinfected with VA1994, only one individual developed mild eye lesions and carried detectable bacterial load on day 3 PI, while all control birds infected with VA1994 developed extended disease (data not shown). All 8 birds that were first exposed to VA1994 and later inoculated with NC2006, however, developed both eye lesions and carried detectable *Mycoplasma gallisepticum* load (Fig. 2). A comparison of the summed eye score and of the summed bacterial loads show that the sequence in which a house finch was exposed to the two strains had a very significant effect. [Mann-Whitney U-test: bacterial load: $n_1=8$, $n_2=12$; $U=0$; $P < 0.0001$; eyescore: $n_1=8$, $n_2=12$; $U=1.50$; $P < 0.0001$].

DISCUSSION.

Birds re-exposed to the more virulent NC2006 *Mycoplasma gallisepticum* strain developed more severe disease than individuals that had recovered from a first infection with that strain. The response to reinfection significantly increased with decreasing virulence of the initial exposure. The response to a heterologous strain, however, differed greatly between reciprocal infections and depended on the sequence in which the birds were exposed. Thus most birds that had recovered from an infection caused by the virulent NC2006 strain were able to clear the infection with the less virulent VA1994 strain almost immediately, while all birds that were first infected with VA1994 and later by the more virulent NC2006 developed disease and maintained *Mycoplasma gallisepticum* loads sufficient for further transmission.

Although the results from both experiments support the hypothesis that response to reinfection is driven by differences in virulence between the first and the second infection, and that only an infection with of a more virulent strain after recovery from a less virulent one results in disease and possible transmission, our reciprocal reinfection experiment with a heterologous strain only involved two *Mycoplasma gallisepticum* strains. To conclusively confirm the generality of this result one would need to use more than two strains, have a fully factorial design whereby all strains would be used both for the initial infection and for the re-infection, and all experiments should be carried out simultaneously.

An implication of this results is that the observed increase in pathogen virulence once the disease became endemic in an area (11) could, at least in part, be driven by increased resistance to reinfection of recovered birds. This follows from the observations that a high proportion of house finches becomes infected with *Mycoplasma gallisepticum* in the fall (1, 2, 9, 12, 15), and a substantial proportion of the birds recover in the wild (5). As a result of that during the fall epidemic that occurs every year successful transmission becomes increasingly more difficult as juveniles recovered from a first *Mycoplasma gallisepticum* infection become more resistant to reinfection (14).

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Abbreviations:

CCU	color-changing units
ELISA	enzyme-linked immunosorbent assay
IACUC	Institutional Animal Care and Use Committee
PI	post infection
qPCR	real-time polymerase chain reaction

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Fig. 1.

Changes in mean bacterial load $\pm$ se, and mean eyescore $\pm$ se of birds reinfected with NC2006 after having recovered from an earlier exposure to one of three MG isolate; control birds were naïve for *Mycoplasma gallisepticum* when infected with NC2006. Inoculum used in 1st infection: Circles: sham infected; triangles: CA2006; squares: VA1994; stars: NC2006. Disease severity upon reinfection increases with increasing difference in virulence between the 1st and the 2nd strain used.

Fig. 2.

Changes in mean bacterial load $\pm$ se, and mean eyescore $\pm$ se of birds reinfected with a heterologous isolate. Circles: 1st inoculation with VA1994, 2nd inoculation with NC2006; triangles: 1st inoculation with NC2006, 2nd inoculation with VA1994. The sequence of exposure to heterologous strains determines the response to infection.

Table 1.

Response of house finches to reinoculation with the NC2006 *Mycoplasma gallisepticum* strain after having recovered from an initial exposure to different isolates (mean $\pm$ se). Groups with different letters (A, B, C, D) differ significantly ($P < 0.05$) from one another following Scheffé's test.

Isolate	Negative Control	CA2008	VA1994	NC2006
N	12	8	8	12
Mean summed	18.51 $\pm$ 1.24	13.43 $\pm$ 1.12	5.88 $\pm$ 1.26	0.00 $\pm$ 0.00
bacterial load to day 35 PI	A	B	C	D
Mean summed	49.75 $\pm$ 5.14	27.13 $\pm$ 6.60	13.00 $\pm$ 2.78	0.17 $\pm$ 0.17
eyescore to day PI 91	A	B	BC	C