Apparent effect of chronic \textit{Plasmodium} infections on disease severity caused by experimental infections with \textit{Mycoplasma gallisepticum} in house finches

André A. Dhondt \textsuperscript{a, *}, Keila V. Dhondt \textsuperscript{b}, Sophie Nazeri \textsuperscript{b}

\textsuperscript{a} Laboratory of Ornithology, Cornell University, Ithaca, NY, 14850, United States
\textsuperscript{b} Department of Microbiology and Immunology, College of Veterinary Medicine, Cornell University, Ithaca, NY, 14850, United States

\section*{1. Introduction}

Chronic infections with \textit{Plasmodium} may adversely impact birds as experimentally shown by effects on reproduction (Merino et al., 2000; Knowles et al., 2010), on feather growth and quality (Marzal et al., 2013) or on telomere–length degradation (Aghar et al., 2015, 2016). \textit{Mycoplasma gallisepticum} is a bacterium that jumped from poultry to house finches (\textit{Haemorhous mexicanus}) in the early 1990s in which it causes severe conjunctivitis (Ley et al., 1996; Hochachka et al., 2013) and can have severe impact on population size (Hochachka and Dhondt, 2000; Dhondt et al., 2006). As it spread across the continent \textit{M. gallisepticum} rapidly evolved (Delaney et al., 2012; Tulman et al., 2012; Hochachka et al., 2013) and increased in virulence once endemic in a region (Hawley et al., 2013). Because haemosporidian parasites are frequently detected in house finches (Kimura et al., 2006; Davis et al., 2013), and given the increasing interest of possible effects of coinfection (Cressler et al., 2016) we tested to what extent chronic \textit{Plasmodium} infections would impact house finches experimentally exposed to \textit{M. gallisepticum}.

The objective of the experiment was to determine if (1) birds naturally infected with \textit{Plasmodium} would become more rapidly infected with \textit{M. gallisepticum} through horizontal transmission compared to individuals in which no \textit{Plasmodium} was detected; (2) if birds infected with both \textit{M. gallisepticum} and \textit{Plasmodium} would develop more severe \textit{M. gallisepticum}-induced clinical disease than birds infected only with \textit{M. gallisepticum}; and (3) to what extent the response would vary with the virulence of the \textit{M. gallisepticum} strain used.

\section*{2. Material and methods}

Juvenile house finches were captured August–October 2015 in Ithaca, Tompkins County, New York (42°46′ N, 76°45′ W) under permit (New York State Fish and Wildlife License 39, Albany, NY; and United States Geological Survey, Department of the Interior, Laurel, MD, permit 22669). Experiments were approved by Cornell University’s IACUC protocol 2009-034.

Only birds negative for \textit{M. gallisepticum} were used. Infection status was determined by visual inspection for eye lesions (Kollas et al., 2004), by Realtime Polymerase Chain Reaction (qPCR) designed to test for the presence of \textit{M. gallisepticum} DNA from conjunctival swabs (Grodio et al., 2008), and by Rapid Plate Agglutination (RPA) to test for the presence of \textit{M. gallisepticum}-specific antibodies in blood (Sydenstricker et al., 2006). We identified haemosporidian infection by lineage using DNA extracted from blood and using the nested polymerase chain (PCR) reactions described by Hellgren et al. (2004) and Waldenstrom et al. (2004) targeting the mitochondrial cytochrome \textit{b} gene for \textit{Plasmodium} and \textit{Haemoproteus}. We repeated the PCR test three times for each...
sample. All PCR products positive for any haemosporidian infection were sequenced and the product compared to the Malavi database (Bensch et al., 2009).

Captive house finches were placed in six groups of 12 individuals housed in large semi-outdoor octagonal aviaries with a ground surface area of 6.87 m² and a volume of 17.87 m³ (Dhondt et al., 2013). Each aviary contained water and a multipierch tube feeder that dispensed ad libitum a pelleted diet (Roudybush, Inc, Cameron Park, CA) (2/3) mixed with sunflower seeds (1/3). One artificial tree and multiple acrylic perches were distributed in identical fashion inside each aviary, as well as ceramic heat lamps. Five of the six groups consisted of six birds with and six without evidence of haemosporidian parasites and one group contained seven birds with and five without haemosporidian parasites.

To introduce *M. gallisepticum* in a group of birds we selected at random one group member with and one without Plasmodium and instilled 0.05 ml of *M. gallisepticum* inoculum in each conjunctival sac. We used three *M. gallisepticum* strains that differed in virulence (Hawley et al., 2013); CA2008 a weakly virulent strain, and NC2008 and CA2015 two more virulent ones. Because the number of colony forming units (CFU) varied between inocula, and as the CFU/ml was lowest for CA2008 at 6.20 \times 10^{6} CFU/ml, we diluted the other inocula with Frey’s medium to equalize the CFU/ml in all three inocula. The precise identifiers for the inocula used are: CA2008-2008.028-2-3P; NC2008-2008.031-4-3P; CA2015-2015.002-3-2P. Each *M. gallisepticum* strain was introduced in two of the groups. The single group in which 5 birds were negative and 7 positive for *Plasmodium* was assigned to CA2008.

We examined the birds for eye lesions, and collected conjunctival swabs for qPCR on day 0, 4, 10, 17, 31, 45, 52, and 59 following the introduction of *M. gallisepticum* in the group on 30 November 2015 (Day 0); we took a blood sample for antibody testing by RPA on Day PI (post introduction) 0, 17, 31, 45, and 59. Eye lesions were scored on a scale of 0 (no lesions visible) to 3 (severe lesions) for each eye (Sydenstricker et al., 2005). Eye scores used were the Logrank test which allows for censored data, and report the $\chi^2$ and P-values of the Logrank test.

To test for effects of the *M. gallisepticum*-strain and of *Plasmodium* on *M. gallisepticum*-load and on eye scores we summed the 8 values of *M. gallisepticum*-load and eye lesions from day 4 to day 59 PI and used these values in a two-way Analysis of Variance (Statistix10 software package). *M. gallisepticum*-load values were log (value + 1)-transformed. We first calculated the model with an interaction term between *Plasmodium* and *M. gallisepticum*-strain. If the interaction term was not significant, we recalculated the ANOVA without it.

3. Results

3.1. Presence of haemosporidians in juvenile house finches

During our initial screening we detected three different *Plasmodium* haplotypes, but no Leucocytozoan nor Haemoproteus. BLASTN results of the sequences identified in the MALAVI database (Bensch et al., 2009) showed that out of 46 haplotypes PADOM11 (*Plasmodium* sp.) was found 35 times (76.1%), WW3 (*Plasmodium* sp.) was found 9 times (19.6%) and SEIAUR01 (*Plasmodium catherinei*) was found twice (4.5%). We distributed the birds among the six groups to equalize the number of birds with each haplotype. Thus in 5 groups five birds were infected with PADOM11 and one bird with WW3; the sixth group, in which we introduced the *M. gallisepticum* CA2008 strain, had five birds with PADOM11, one with WW3 and one I bird with SEIAUR01.

3.2. Response of index birds to *M. gallisepticum* inoculation

The index birds are the initial source of the horizontal transmission in each group, although as other birds become infected with *M. gallisepticum*, these can also contribute to further transmission. To provide an idea about differences between the *M. gallisepticum* strains used in this experiment, and between birds with and without *Plasmodium* we summarize the mean summed *M. gallisepticum*-load across the 8 samples taken on days 4–59 PI for each index bird as shown in Table 1. In all aviaries there was a source of *M. gallisepticum* infection, although in one of the aviaries in which NC2008 was introduced only one index birds developed disease. Given the very small sample sizes we did not perform statistical tests to compare *M. gallisepticum*-load between isolates or birds with/without *Plasmodium*, although the data suggest that the three *M. gallisepticum* isolates differ in virulence as expected (CA2008 < NC2008 < CA2015) and that birds with *Plasmodium* developed infections with a higher *M. gallisepticum*-load and tended to have more severe lesions.

3.3. Horizontal transmission and *Plasmodium* infection

3.3.1. Time to first infection

To determine the time to first infection through horizontal transmission of each naïve individual we determined what day post

| Table 1 | Mean and standard errors of *M. gallisepticum*-load summed to day 59 PI in index birds. |
|---------|---------------------------------|
|          | CA2008 | NC2008 | CA2015 |
| No *Plasmodium* (n = 2) | 17.62 ± 1.17 | 19.26 ± 1.52 | 15.99 ± 2.05 |
| *Plasmodium* (n = 2) | 15.74 ± 9.80 | 21.65 ± 21.65$^a$ | 33.08 ± 5.04 |

$^a$ One of the index birds, although it seroconverted, did not develop disease and hence would not have transmitted *M. gallisepticum*. The summed *M. gallisepticum*-load of the 2nd bird in the same avairy was 43.29.
Table 2
Mean and standard errors of eye scores (eye score) and *M. gallisepticum*-loads (MG load) summed to day 59 PI of house finches exposed to different *M. gallisepticum* isolates that had (Yes) or did not (No) have chronic *Plasmodium* infections before the start of the experiment. Groups of 12 birds were held in large aviaries into which *M. gallisepticum* was introduced. Sample sizes vary between groups because only birds that developed an infection are included in the analysis.

| MG isolate | Plasmodium present | n   | Eye score | MG load |
|------------|--------------------|-----|-----------|---------|
| CA2008     | No                 | 6   | 2.83 ± 1.25 | 1.53 ± 1.48 |
| CA2008     | Yes                | 4   | 1.00 ± 0.58 | 5.14 ± 5.10 |
| NC2008     | No                 | 9   | 7.11 ± 2.34 | 7.11 ± 2.03 |
| NC2008     | Yes                | 7   | 11.43 ± 4.46 | 14.47 ± 4.92 |
| CA2015     | No                 | 9   | 12.11 ± 3.54 | 18.73 ± 2.24 |
| CA2015     | Yes                | 9   | 24.89 ± 3.62 | 27.63 ± 2.57 |

Analysis of Variance Table for *M. gallisepticum*-load summed to day 59 PI

| Source        | DF | SS    | MS | F    | P     |
|---------------|----|-------|----|------|-------|
| Plasmodium    | 1  | 557.91| 557.91 | 5.79  | 0.020 |
| Isolate       | 2  | 2724.89 | 1362.44 | 14.15 | 0.0000 |
| Error         | 40 | 3851.63 | 96.29 |       |       |
| Total         | 43 |       |      |      |       |

Analysis of Variance Table for eye score summed to day 59 PI

| Source        | DF | SS    | MS | F    | P     |
|---------------|----|-------|----|------|-------|
| Plasmodium    | 1  | 453.43| 453.43| 5.20  | 0.028 |
| Isolate       | 2  | 1706.66 | 853.330 | 9.79  | 0.0003 |
| Error         | 40 | 3483.97 | 87.149 |       |       |
| Total         | 43 |       |      |      |       |

introduction (PI) of *M. gallisepticum* in the aviary each individual developed any sign of infection: eye lesions (eye score >0), *M. gallisepticum*-DNA present in the eye swab, or evidence of presence of antibodies if neither other sign of infection was observed.

Survival analysis showed that the three *M. gallisepticum* strains differed significantly in transmission rates \( \chi^2 = 13.32, df = 2, P = 0.0013 \). Transmission was significantly slower in the group with CA2008 than in both other groups (NC2008 versus CA2008: \( \chi^2 = 8.39, df = 1, P = 0.004 \); CA2015 versus CA2008: \( \chi^2 = 8.20, df = 1, P = 0.004 \), but there was no significant difference between CA2015 and NC2008 (\( \chi^2 = 0.28, df = 1, P = 0.60 \)). The lower transmission in the groups exposed to CA2008 was primarily caused by the fact that by day 59 PI, when the experiment was terminated, half of the naive birds still showed no signs of having become infected, while in the other groups more birds showed evidence of having been infected by *M. gallisepticum*.

While the time to infection did differ between groups exposed to different *M. gallisepticum* isolates, there was no effect of *Plasmodium*: birds with and without *Plasmodium* did not differ in time to infection in any of the groups (all \( P > 0.13 \)).

3.3.2. Severity of disease if infected

Some birds did not develop any signs of infection. Because we wanted to address the question how birds responded if infected by horizontal transmission we used only those birds that either developed eye lesions or in which *M. gallisepticum*-load in the conjunctiva was non-zero. This reduced the sample sizes to 10/20 for CA2008, 16/20 for NC2008 and 18/20 for CA2015.

In order to answer questions 2 (effect of *Plasmodium*) and 3 (effect of *M. gallisepticum* strain used?) we performed a two-way analysis of variance with interaction to determine if either *M. gallisepticum*-strain or the presence of *Plasmodium* impacted *M. gallisepticum*-load and/or eye lesions. Given that the interaction term was not significant for *M. gallisepticum*-load (\( P = 0.80 \)) nor for eye lesions (\( P = 0.12 \)) we only report the results without interaction (Table 2) and illustrate the change of *M. gallisepticum*-load and eye lesions for one *M. gallisepticum*-strain over time in Fig. 1. The results show that the response to infection through horizontal transmission in a group varied significantly between *M. gallisepticum*-strains to which the birds were exposed (\( P < 0.0001 \)) but also that birds with a chronic *Plasmodium* infection developed more severe clinical disease (\( P < 0.03 \)).

4. Discussion

House finches chronically infected with *Plasmodium* spp. and exposed to *M. gallisepticum* are equally likely to become infected with *M. gallisepticum* through horizontal transmission than group members without a chronic *Plasmodium* infection. A chronic *Plasmodium* infection thus does not make a bird more sensitive to
infection. In this experiment one of the two birds that were the source of M. gallisepticum in each group had and one did not have a chronic Plasmodium infection. In order to determine the role of chronic Plasmodium infections on M. gallisepticum transmission one would need to compare groups in which both birds used to introduce M. gallisepticum in the aviary did or did not have chronic Plasmodium infections. The latter would deposit more M. gallisepticum on the feeder, which would have an effect on M. gallisepticum transmission rates (Adelman et al., 2013).

Although the Plasmodium-positive birds developed more severe clinical disease in response to the M. gallisepticum exposure, this could be attributed to other traits that correlate with both maintenance of a chronic Plasmodium infection and a stronger response to M. gallisepticum infection, and therefore this experiment, although suggestive, does not conclusively prove cause and effect. For example, social status or multilocus heterozygosity by influencing immune competence (Hawley et al., 2005, 2007) might alter the response to both infections. Experiments in which birds, naïve to both pathogens, are experimentally infected with Plasmodium and with M. gallisepticum are needed to confirm that the relationship found is causal.

Dhondt and Dobson (2017) speculated that in birds infected both with the widespread Plasmodium parasite and the emerging M. gallisepticum bacteria coinfection might facilitate transmission of both (Dhondt and Dobson, in press). Thus, if we can confirm that birds coinfected with Plasmodium carry higher M. gallisepticum loads for a longer period of time this could lead to higher transmission rates (Hawley et al., 2013; Williams et al., 2014) resulting in a higher proportion of the population being infected with M. gallisepticum. The observation that prevalence of mycoplasmal conjunctivitis increases from North to South among house finch population in eastern USA (Altizer et al., 2004) might in part be explained by higher Plasmodium parasitaemia in those regions (Davis et al., 2013). Reciprocally because house finches infected with M. gallisepticum transiently increase corticosterone levels (Love et al., 2016), they are more attractive to mosquitoes (Gervasi et al., 2016). The increased mosquito feeding rate could facilitate Plasmodium transmission. This remains to be tested experimentally.

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Appendix A. Supplementary data
Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ijppaw.2017.03.003.

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