**Experimental test of virulence-driven trade-off in an emerging disease.**

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**Short running title:** experimental test of virulence-driven trade-off

**Abstract**

**Keywords:**

A central hypothesis to explain what drives changes in virulence when a pathogen infects a novel host is the *virulence-transmission hypothesis,* further VTH (Anderson and May 1982). This hypothesis postulatesthat virulence, or the cost imposed on the host by the pathogen, should also result in a cost to the pathogen itself in terms of pathogen transmission. This is because an increase in virulence would cause an increase in host mortality thereby reducing the duration of the transmission period during which the parasite can infect other host individuals (Alizon et al. 2009). The VTH suggests that this effect is non-linear and that as within-host replication increases virulence also increases and transmission rate decelerates because the duration of the infectious period declines (Acevedo et al. 2019). The outcome predicted by the VTH is a stabilization of the pathogen-host interaction at intermediate pathogen virulence.

In a recent meta-analysis to explore the extent of the support for the VTH in host-pathogen systems Acevedo et al. (2019) explored the extent of the empirical support for four key relationships that are predicted under the VTH. Only two were supported by existing research.

They found:

* strong support for a positive relation between *pathogen replication and virulence*, but the expected polynomial term that is required to represent a deceleration in virulence with increasing replication was not statically significant.
* similarly, they found a strong increasing relationship between *pathogen replication and transmission*, but again the decelerating term was not statistically significant.
* As regards the two other predicted relationships, they did not find statistically significant support for a relationship between *virulence and transmission*, nor between *recovery and virulence*, and concluded that more and better planned studies are required to test the Anderson and May hypothesis.

In a commentary on Acevedo’s paper Hector and Booksmythe (2019) concluded that “*Little evidence exists for a virulence-transmission trade-off*”. Their paper concludes that linking within-host dynamics (virulence, replication and recovery) to between-host dynamics is important for understanding host-pathogen dynamics.

One of the systems that is reported in Acevedo’s review is the House Finch-*Mycoplasma gallisepticum* system (further HF\_MG system). This system originated when the widespread bacterial poultry pathogen *Mycoplasma gallisepticum* (*M. gallisepticum*) jumped from poultry to house finches *Haemorhous mexicanus* (and other wild birds) in the early 1990s thereby expanding its tissue tropism: in poultry *M. gallisepticum*  causes disease mainly in the respiratory tract, while in house finches it also causes disease in the conjunctiva. In house finches virulence is related to conjunctival load not to choanal load (Reinoso-Perez et al. 2022). In their meta-analysis Acevedo et al. reported a significant relationship between pathogen load and virulence (Hawley et al. 2013), and between virulence and transmission (Williams et al. 2014) for the HF\_MG system. Over a 20 year period Bonneaud and Longdon (2020) collected a large number of *M. gallisepticum* strains from house finches in Alabama and neighboring states and used 55 of these to test aspects of the VTH hypothesis. They concluded that their study supported the VTH as they found a positive association between virulence and transmission, and also that pathogen fitness was highest at intermediate virulence.

What is important about the HF\_MG system is that it originated following a natural host jump from poultry (Ley et al. 1996), was studied since its discovery in 1994 (Fischer et al. 1997, Dhondt et al. 1998, Nolan et al. 1998), and that as it rapidly spread across eastern North America no efforts were made to control it. During its expansion *M. gallisepticum* virulence rapidly increased in eastern North America (Hawley 2013). After having spread across the eastern USA a second epidemic started when a novel derived variant of  *M. gallisepticum* successfully spread to Oregon in 2004 (Ley et al. 2006, Hochachka et al. 2013) and from there to California (Dhondt et al. 2006). Because our team was already studying *M. gallisepticum* in wild birds we were primed to collect a time series of *M. gallisepticum* samples in California starting with an isolate of very low virulence in 2006. *M. gallisepticum* virulence in California rapidly increased over the next 10 years (Hawley et al. 2013).

In this paper we report on experiments in which we test the VTH by exposing groups of house finches kept in large aviaries in which we measured pathogen within-host replication, pathogen virulence, time to recovery and transmission in semi-natural conditions using five California isolates that differ in virulence (Dhondt et al. 2012). We describe the relationships between replication and virulence, replication and transmission, virulence and transmission and virulence and duration of infectious period. The product of transmission rate and duration of infectious period was used as a proxy of pathogen fitness which allowed us to relate virulence to fitness.

***Material and Methods***

**Origin of the birds**

All birds used in the experiments described in this paper were juvenile house finches captured in the fall in Ithaca, Tompkins County, New York (42°46’ N, 76° 45’ W) under permit (New York State Fish and Wildlife License 39, Albany, NY; 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 not evidencingcurrent or previous *M.* *gallisepticum* infection after having been kept in quarantine for a minimum of two weekswere used. Infection status was determined by visual inspection for eye lesions (Kollias et al. 2004), by Realtime Polymerase Chain Reaction (qPCR) designed to measure the bacterial load of *M. gallisepticum* DNA from conjunctival swabs (Grodio et al. 2008), and by Rapid Plate Agglutination (RPA) to test for the presence of *M. gallisepticum*-specific antibodies in blood (Sydenstricker et al. 2006). To be considered naïve for *M. gallisepticum* birds needed to test negative for all three criteria following a 2 -week quarantine period.

**Experiments with *M. gallisepticum* isolates**

Between 2010 and 2016 we carried out a series of identical experiments called to compare virulence and transmission rates of different isolates. As in this paper we only use results using California isolates we only report those in Table 1. Birds were kept in groups of 12 *M. gallisepticum*-naïve, wild caught, juvenile house finches. Each group was housed in one of six large aviaries of 17 m3 each (Dhondt et al. 2012) inside a large closed barn. All birds were individually color-banded. In each group, two finches were chosen at random to be the source of the *M. gallisepticum* introduction into a group (hereafter called “index birds”). Index birds were inoculated with 50 µl of inoculum in each eye using a micropipette; they were then kept in a paper bag for 10 minutes to allow inoculum absorption, after which they were released back into their caged flock. The other 10 birds in the group are called sentinel birds. The inocula used in the experiments are listed in Table 1. The experiments with isolates CA2006, CA2009, and CA2010 lasted for 70 days, and those with CA2008 and CA2015 lasted for 59 days. The longest duration of any infectious period was 59 days for 2 birds inoculated with CA2009.

[With the exception for birds inoculated with CA2006, in which not all birds developed eye lesions and the first day on which eye lesions were detected was 4, 10, 12, 18, and one bird inoculated with isolate CA2009, all other index birds had developed eye lesions on DPI 4].

Table XX. Details of experiments. Each treatment was replicated using two groups of 12 birds, two of which (index birds) were inoculated in each eye with 50 µl of inoculum. In each set the inoculum was diluted with Frey’s medium so that all inocula had the same concentration. The detailed origin of the isolates is reported in (Ley et al. 2016). All isolates were obtained in California (represented by its two first letters) followed by and the year in which it was obtained.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Isolate and experiment | Inoculation date | Initial days sampled | Isolate number, passage, date | Original CFU/ml | Dilution factor and resulting log MG | Log # MG inserted in both eyes |
| CA2006 | 4-Feb-10 | 4,11\*,18\*,25 | 2006.052-5- 4p 1/13/2009 | 3.04 x 106 | target 6.48 | 5.48 |
| CA2006 | 1-Nov-13 | 5,11,19,25 | 2006.052-5 (4p) 1/13/09 | 3.04 x 106 | target 6.48 | 5.48 |
| CA2009 | 1-Nov-13 | 5,11,19,25 | 2009.061-1 (3p) 10/25/10 | 1.87 x 107 | 1:6.15 6.48 | 5.48 |
| CA2010 | 1-Nov-13 | 5,11,19,25 | 2010.003-1 (3p) 10/25/10 | 4.60 x 107 | 1:15.1 6.48 | 5.48 |
| CA2008 | 30-Nov-15 | 4,10,17,31 | 2008.028-2-(3p) 11/19/10 | 6.20x 106 | target 6.20 | 5.6 |
| CA2015 | 30-Nov-15 | 4,10,17,31 | 2015.002-3-(2p) 2 8/16/12 | 2.80 x 108 | 1: 45 6.20 | 5.6 |

\* An asterisk following the sampling day means that half of the birds were sampled that day, and the other half the following day. CA2006 was used in two different years as an internal control

Inocula were grown and their CFU quantified in David Ley's lab (NCSU) (as described in Ley et al. 2016), placed in 1 ml vials, and stored at -80°C. As a measure of bacterial load we use CFU (colony forming units) which for *Mycoplasma spp.* is very similar to CCU (color changing units) (Stemke and Robertson 1982, Assuncao et al. 2006). The number of bacteria in each sample was determined as a CFU/ml before freezing and shipping to Ithaca. In Ithaca the inocula were stored at -80°C until thawed just before inoculation.

As the inocula for CA2009, CA2010 and CA2015 were one or two orders of magnitude higher than those of CA2006 and CA2008 they were diluted using Frey's medium, so that the number of bacteria instilled in the eyes were similar between all inocula. As we introduced 50 µl of inoculum (1/20 of 1 ml) in each eye of a bird we estimated bacterial load on day zero of an experiment as 1/10th of the concentration of the inoculum as indicated in Table 1.

Index birds were infected on following dates: 4 February 2010 (CA2006) ; 1 November 2013 (CA2006, CA2009, CA2010); 30 November 2015 (CA2008, CA2015). After infection all birds were recaptured at regular intervals and tested for infection by *M. gallisepticum* by (1) visual inspection for eye lesions (Kollias et al. 2004, Sydenstricker et al. 2006), (2) by qPCR designed to measure the bacterial load of *M. gallisepticum* DNA from conjunctival swabs (Grodio et al. 2008), and/or (3) by RPA to test for the presence of *M. gallisepticum*-specific antibodies in blood (Sydenstricker et al. 2006). As in previous experiments we had found that following infection many birds did not test positive for all three tests (Sydenstricker et al. 2005, Sydenstricker et al. 2006, Dhondt et al. 2012) a bird was considered infected if it was positive for any of the tests. Each isolate was replicated twice, but isolate CA2006 was used twice to test if birds born in different years but infected with the same isolate would respond differently to infection. As the survival rate of the index birds inoculated with CA2006 yielded no difference (Logrank test: CA2006 P=0.75) we combined the results of these experiments in further analyses.

**Calculation of demographic variables**

We defined **virulence** (= severity of eye pathology) as the the eye scores in both eyes combined summed over the first three observations days following inoculation (day 4; day 10 + 1 day; day 18 + 1 day; further, for brevity, day 4, 10, 18).

We defined r**eplication** as the log-transformed bacterial loads in both eyes combined summed over the first three observations days following inoculation (day 4; day 10 + 1 day; day 18 + 1 day; further, for brevity, day 4, 10, 18).

**Duration of infectious period**. House finches respond to *M. gallisepticum* infection not only by developing eye lesions but also by developing fever (Hawley et al. 2012), lethargy, becoming inactive (Dhondt KV et al. 2007), and reduced avoidance behavior (Dhondt KV et al. 2007, Adelman et al. 2015, Bonneaud et al. 2020). The severity of conjunctivitis in an eye was measured on a scale from zero (no clinical signs) to 3 very severe conjunctivitis (Sydenstricker et al. 2006). If both eyes had a score of 3 birds were lethargic and functionally blind and hence easier to capture (Bonneaud et al. 2020). In captivity birds usually recovered eventually, but in the wild diseased birds had a reduced survival probability (Faustino et al. 2004) resulting in a decline in population size (Hochachka and Dhondt 2000, Nolan et al. 1998). For that reason, and following Bonneaud (2020), we considered that a bird had died the day following an eyescore of 6.The duration of the infectious period was calculated between the 1st day on which a bird had an eyescore >0, to the day following an eyescore of 6. If the eyscore did not reach 6 during the experiment then the duration of the infectious peiod was the number of days between the first date on which we observed an eyescore, to the first observation day on which the eyscore had returned to zero. The longest infectious period in any bird was 55 days.

**Transmission rate.** Transmission between the index birds and the 10 sentinel birds in the same aviary was measured by trapping all birds on day 4 PI, and then every week and testing them for infection by the three methods described above. We combined the data from all replicates of the same isolate to calculate a single value for mean time to infection per *M. gallisepticum* isolate. We calculated transmission rate per week using a Bayesian transmission rate analysis as described in the Supplementary Material.

For the Bayesian analysis, we also combined the data from the two replicates for each isolate. For each strain, the data entering into the analysis are

* The numbers of uninfected and infected birds in an aviary at censuses *k*=1, 2, 3*,...* in aviary *j*, which we denote **U**(*j*, *k*) and **I**(*j, k*), respectively.
* The number of new cases **C**(*j,k*) between census *k* and census (*k*+1) in aviary *j*, i.e., the number of initially susceptible birds that were found to be infected at the next census.

We assumed that all new cases between two successive censuses arose from direct infection of an initially uninfected bird by an initially infected bird and that the instantaneous force of infection λ (i.e., the probability per unit time of becoming infected) is proportional to the number of initially infected birds,

λ(*j,k*) = β**I**(*j,k*). (S.1)

The constant of proportionality β measures the infectivity of the strain, and is the parameter we wish to estimate. The units of β are (wk-1 individuals-1) so that the units of λ are wk-1.

The probability that a bird uninfected at census *k* is still uninfected at census (*k*+1) is then

exp(-β**I**(*j,k*)τ(*j*,*k*)) where τ(*j*,*k*) is the time (in weeks) between census *k* and census (*k*+1) in aviary *j*. The probability of becoming infected is thus 1­­- exp(-β**I**(*j,k*)τ(*j*,*k*)). Assuming that the fate of each bird in the aviary is independent, the total number of cases during the week is distributed as

**C**(*j,k*) ~ Binomial(N=**U**(*j*,*k*), p = 1- exp(-β**I**(*j,k*) τ(*j*,*k*))). (S.2)

Equation (S.2) is the statistical model that was fitted to the data on each isolate (all census pairs, in both aviaries) to estimate the value of β.

Estimation was done in a Bayesian framework, using the **jagsUI** package version 1.5.2 (Kellner 2021) in **R** version 4.1.1 (R Core Team 2021). **jagsUI** uses the JAGS software (Plummer 2021) to perform Bayesian inference using Markov Chain Monte Carlo (MCMC). The prior distribution on β was extremely vague, specifically log(β) was taken to have a Gaussian distribution with variance 106. MCMC chains were initialized at a method of moments estimate of β (details are given below). For each isolate we ran 5 independent MCMC chains, with 5000 burn-in iterations followed by 200,000 iterations that were thinned by a factor of 4 to remove autocorrelation, so that inference for each isolate was based on 250,000 effectively independent samples from the posterior distribution. Because only one parameter was being estimated, the computing time for this many samples was under a minute per isolate.

Visual inspection of trace plots confirmed that the chains had achieved stationarity by the end of the burn-in period, and convergence was additionally assessed using the Gelman-Rubin statistic , which had reported value 1.000 (yes, that is actually what happened) for both β and the deviance for all isolates. Without thinning, the lag-1 autocorrelation coefficients in the posterior samples were as large as 0.2 for some isolates. The autocorrelation function of the thinned samples confirmed that four-fold thinning was sufficient to effectively remove autocorrelation (specifically, autocorrelation coefficients at lags 1-20 were uniformly below 0.02 in absolute value for all chains for all isolates).

The method of moments estimate for each isolate was as follows. For every pair of successive censuses in both aviaries, the Binomial random variable on the right-hand of eqn. (S.2) was replaced by its expectation, and the resulting equation was numerically solved for β. To avoid numerical problems, in a few cases where **C**=**U** (i.e., all uninfected birds became infected) we replaced **U** by **U**+0.5. These separate estimates for β for each census pair were then averaged to get an overall estimate that was used to initialize each MCMC chain.

**Pathogen fitness**

To estimate pathogen fitness we multiplied the duration of the infectious period with the transmission rate

**Statistical analyses (to be corrected)**

*Relationship between replication and virulence*

To test if *replication and virulence* were correlated we fitted a mixed model to the data using BACTERIAL LOAD (log qPCR; fixed effect, continuous variable), the log-transformed *M. gallisepticum* load determined with qPCR summed over the three first sampling periods; we included the random intercept, ISOLATE (random effect) as well. The names of the variables are in upper case.

*Relationship between virulence and transmission.* We fitted a single model in order to determine the characteristics of the bacterial isolates that led to different efficiencies of transmission. As our index of efficiency of transmission, we used the probability that an experimental house finch became infected by day 18 PI. The predictor variables that we examined were the region from which the isolate was obtained (REGION: Eastern or Western), sum of the eye scores of the two index (i.e. experimentally infected) birds (DISEASE SEVERITY), and the interaction between REGION and DISEASE SEVERITY. The model was fitted using the *glmer* function of *lme4* using a binomial error (i.e. fitting a logistic regression model). We represented each experiment’s data as a single data record, while still treating each bird’s disease state (infected or uninfected) as a separate piece of information. We did this by inputting the response information into the analyses as a two-column matrix in which the first column was the count of house finches within the experiment that were infected during the time period up to and including day 18 PI, and the second column was the count of the birds within each experiment that were uninfected by day 18 PI. We also statistically accounted for the potential for idiosyncratic differences in transmission among the experiments by including experiment as a random intercept term in the model. We calculated the probabilities of statistical significance of effects using likelihood ratio tests, because the p-values associated with the coefficient estimates and their standard errors are testing the hypotheses that the coefficients do not differ from zero, which for logistic regression is a test of whether the coefficients are describing probabilities that differ from 0.5 and not whether the predictor variable has zero effect on the response. We used R’s *anova* function to calculate the probabilities of significant increase in explanatory power when each predictor variable was added to the model. The probabilities associated with REGION and DISEASE SEVERITY were calculated by comparing the maximum likelihoods of an intercept-only model with a model containing the added effect of either REGION or DISEASE SEVERITY, respectively. The probability associated with the interaction was a comparison of the model containing the two main effects, and the model containing the two main effects plus their interaction. When graphing the patterns predicted by the full model, we bootstrapped the 95% confidence limits around predictions using the *bootMer* function from the R package *lme4*, based on 10000 bootstrap simulations in which separate random effects were simulated for each iteration and using a parametric bootstrap process.

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**Isolates used**

The difference between western isolates, however, was very significant:

Kruskal-Wallis Statistic, corrected for ties : H= 13.78

P-Value, Using Beta Approximation 0.0012

**isolate N Mean SE**

CA2006 8 -3.4038 0.6474

CA2008 4 -0.3775 0.9156

CA2009 4 0.9725 0.9156

CA2010 4 0.9775 0.9156

CA2015 4 0.1225 0.9156

**One-Way AOV for change by isolate**

**Source DF SS MS F P**

isolate 4 83.494 20.8735 6.22 0.0022

Error 19 63.717 3.3535

Total 23 147.211

The LSD a-posteriori test shows that the change in pathogen load with CA2006 is significantly different from all othe western isolates; the latter do not differ significantly from one another

(**LSD All-Pairwise Comparisons Test of change by isolate**

**isolate Mean Homogeneous Groups**

CA2010 0.9775 A

CA2009 0.9725 A

CA2015 0.1225 A

CA2008 -0.3775 A

CA2006 -3.4038 B

Alpha 0.05 Standard Error for Comparison 1.1214 TO 1.2949

Critical T Value 2.093 Critical Value for Comparison 2.3472 TO 2.7103

There are 2 groups (A and B) in which the means

are not significantly different from one another.

The Tukey HSD a-posteriori test indicate that CA2006 differs from CA2009, CA2010, CA2015, but not from CA2008. The latter also does not does differ from the 3 later western isolates. differfrom

**Tukey HSD All-Pairwise Comparisons Test of change by isolate**

**isolate Mean Homogeneous Groups**

CA2010 0.9775 A

CA2009 0.9725 A

CA2015 0.1225 A

CA2008 -0.3775 AB

CA2006 -3.4038 B

**Pathogen replication and virulence**

The relationship between replication and virulence is significant, but does not decrease with increasing MG replication. Eastern isolates have a higher virulence for the same pathogen load compared to derived western isolates. This result is similar to the results reported by Acevedo’s meta-analysis (Acevedo et al. 2019).

1. **Replication and transmission**

Transmission increased with replication (summed to DPI 18). Again we find a significant difference between eastern and western isolates. Transmission increases with replication but the graph does not show a decelerating relationship, a result similar to that in Acevedo’s meta-analysis. Western strains the isolates with the highest replication have a lower transmission rate than the isolate with an intermediate replication.

1. Vi**rulence and transmission**

The relationship between virulence and transmission is very similar to that for that between replication and transmission: highly significant, not decelerating and different between eastern and western isolates.

1. **Recovery and virulence**

Given that in the wild, although some birds recover, a *M. gallisepticum* infection reduces house finch survival (Faustino et al. 2004), and that we can assume that survival decreases with the severity of the eye lesions and associated changes in behavior (Kollias et al. 2004, Hawley et al. 2007), we followed (Bonneaud and Longdon 2020) and used as a proxy for disease-induced mortality the severity of the eye lesions. They observed that when a bird had severe eye lesions (in our study a score of 6), and their avoidance behavior to hand capture was reduced the birds could be considered to have died . We considered a bird to have died on the day following the day on which they had an eyescore of 6, because such an eye scores were associated with a severe reduction in activity (Dhondt et al. 2007). We defined as *duration of infection* the number of days between the first day on which an eyescore >0 was observed and either the first day on which the bird had recovered or the observation day following the day on which the eyescore was of 6.

Given the large differences of infection with eastern and western strains we analyzed the data separately (Fig. xx). The time to recovery varied with virulence. In western strains time to recover was the highest for the strain with intermediate virulence.

1. Virulence and “fitness”

To approximate pathogen fitness we have multiplied transmission rate (beta) with duration of infection.



**Transmission without disease**

In our experiments we used three measure of infection: elevated antibody levels, the presence of M. gallisepticum DNA in eye swabs, and eye inflammation. In some experiments we observed that asymptomatic carriers could transmit, and that the extent the proportion of asymptomatic carriers correlated strongly with the transmission rate.

Discussion

Our results are similar to those summarozed by Acevedo in that the relationships between pathogen replication and virulence, and between replication or virulence and transmisison are positively correlated without a clear indication of the predicted decrease at yteh highest vruence, but different in that time to recovery first increases, but then decreases with virulence, resulting from a severy increase in apparent mortality with virulence.

If we then calculate a proxy for pathogen fitness by multiplying transmisison rate with the duration of the period during which transmisison occurs we find similar curvivlinear relationship.

Our data, therefore, support the VTH in that the optimal virulence from the pathogen point of view is intermediate.



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