EVOLUTION

Incomplete host immunity favors the evolution of virulence in an emergent pathogen

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Immune memory evolved to protect hosts from reinfection, but incomplete responses that allow future reinfection may inadvertently select for more-harmful pathogens. We present empirical and modeling evidence that incomplete immunity promotes the evolution of higher virulence in a natural host-pathogen system. We performed sequential infections of house finches with *Mycoplasma gallisepticum* strains of various levels of virulence. Virulent bacterial strains generated stronger host protection against reinfection than less virulent strains and thus excluded less virulent strains from infecting previously exposed hosts. In a two-strain model, the resulting fitness advantage selected for an almost twofold increase in pathogen virulence. Thus, the same immune systems that protect hosts from infection can concomitantly drive the evolution of more-harmful pathogens in nature.

Imperfect vaccines promote the evolution of more-harmful pathogens by creating a fitness advantage for virulent strains in vaccinated hosts, such as high rates of infectivity or transmission (*1*–*5*). By preventing disease-induced host death and subsequent removal of virulent strains from a population, imperfect vaccines also reduce the overall costs of virulence to pathogens (*5*). We asked whether incomplete immune responses to natural infections can similarly favor the evolution of more-virulent pathogen strains that cause greater host mortality. The bacterial pathogen *Mycoplasma gallisepticum* emerged in the 1990s in free-living house finches (*Haemorhous mexicanus*), causing severe conjunctivitis that indirectly reduces finch survival via visual impairment and reduced ability to escape predators (*6*, *7*). After emergence in the eastern United States, *M. gallisepticum* spread throughout the house finch range (*8*), transmitted by direct contact and contaminated surfaces such as bird feeders (*9*). Soon after the pathogen became endemic on each coast of the United States, the virulence of circulating *M. gallisepticum* strains rapidly increased, as measured by disease severity produced in immunologically naïve hosts (*10*). More than half of free-living house finches can recover from *M. gallisepticum* infection (*6*), generating pools of recovered hosts in wild populations. Furthermore, recovered hosts show strong but incomplete immune protection and thus can become reinfected with homologous or heterologous pathogen strains (*11*–*13*). In our study, we tested whether incomplete host immunity drives the evolution of greater virulence in this system.

We used sequential-inoculation experiments to quantify how incomplete immunity generated from experimental prior exposure alters host responses relevant for pathogen fitness. House finches naïve to *M. gallisepticum* at capture (n = 120 finches) were individually housed and sequentially exposed to pairs of *M. gallisepticum* strains that either were identical (homologous) or had different levels of virulence (heterologous), with clinical recovery between exposures. We performed two identical experiments in successive years, each using three distinct pathogen strains in a completely randomized design (Tables 1 and 2). Positive controls received sterile medium during primary inoculation and thus had no pathogen exposure before secondary inoculation with one of the six strains. We quantified strain virulence as the degree of within-host replication (ε in Tables 1 and 2) using conjunctival pathogen loads measured by quantitative polymerase chain reaction (qPCR) (*10*) across 8 weeks after primary inoculation of immunologically naïve birds (see supplementary materials). To examine how the virulence of the primary and secondary strains affected host responses relevant for pathogen fitness, we measured conjunctivitis severity and pathogen load for 5 weeks after secondary inoculation. Conjunctivitis severity, which correlates with disease-induced mortality risk in the wild (*6*, *7*), was scored on an ordinal scale from 0 to 3, and conjunctival pathogen load was quantified by qPCR (*10*).

First, we found that hosts with prior experimental exposure to any *M. gallisepticum* strain showed reduced severity of the clinical signs that predict mortality risk in the wild (*6*, *7*) compared with hosts with no prior exposure (Fig. 1A). Thus, consistent with findings from earlier work using vaccines (*5*), host immunity reduced the costs of virulence to the pathogen by protecting hosts

Table 1. Relative virulence and $\log_{10}$ conjunctival pathogen loads (means ± SEM) for experiment one. All birds received primary and secondary inoculations with either sterile medium or one of three *M. gallisepticum* strains that varied in virulence (*10*). Strains are ordered from low to high virulence (left to right and top to bottom), and relative virulence terms describe the primary exposure strain relative to the secondary exposure strain (e.g., "less virulent" indicates primary exposure to a less virulent strain than the secondary strain). Numbers in column headings indicate strain virulence estimated from a separate data set for naïve hosts (see supplementary materials). Strain virulence values (ε) are linear model coefficients (for a linear mixed-effects model fitting strain effects in naïve birds using data from all experimental strains; likelihood ratio = 172.56, df = 6, 124, $P < 0.001$), whereas values in the table are raw means of pathogen load (n = 4 to 5 birds for each group). Pathogen loads tend to increase top to bottom (with increasing virulence of the secondary strain) but decrease left to right within a row (with increasing virulence of the primary strain). N/A, not applicable.

	Relative virulence and mean pathogen load for primary exposure			
Secondary exposure	Sterile medium (control)	CA2006 low (ε = 1.41 ± 0.34)	VA1994 intermediate (ε = 3.31 ± 0.34)	NC2006 high (ε = 4.72 ± 0.34)
CA2006	No prior exposure 1.54 ± 0.23	Homologous 0.47 ± 0.17	More virulent 0.20 ± 0.13	More virulent 0.27 ± 0.14
VA1994	No prior exposure 3.29 ± 0.35	Less virulent 1.11 ± 0.31	Homologous 0.62 ± 0.20	More virulent 1.10 ± 0.18
NC2006	No prior exposure 4.71 ± 0.52	Less virulent 3.30 ± 0.39	Less virulent 1.78 ± 0.18	Homologous 1.02 ± 0.17
Sterile medium (control)	Negative control 0.044 ± 0.027	N/A	N/A	N/A

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from the disease-induced mortality that prevents onward pathogen transmission and thus reduces pathogen fitness to zero. Second, we observed the greatest reduction in pathogen load and clinical signs and, thus, the strongest protection against reinfection in hosts previously exposed to higher-virulence strains (Fig. 1 and Tables 1 and 2). Primary exposure to a homologous strain also generated strong host protection (Fig. 1), suggesting that adaptive immune responses, either alone or in combination with innate priming mechanisms (*14*), likely underlie the detected incomplete protection against reinfection (see also fig. S1).

We used the empirical responses to secondary inoculation to fit two key pathogen-associated traits, disease-induced mortality and susceptibility, as continuous functions of the virulence of both the primary and secondary strains (see supplementary materials). Disease-induced mortality rates were inferred to scale linearly with conjunctivitis severity, which predicts mortality risk in the wild (*7*); susceptibility to infection was inferred from the presence of a pathogen load above 10^2 copies in the conjunctiva at any post-inoculation qPCR sampling point. As expected, disease-induced mortality and susceptibility increased with the virulence of the currently infecting strain (Fig. 2 and tables S1 and S2). Thus, virulent strains are better able to successfully infect hosts but also cause higher disease-induced mortality than less virulent strains. However, the host immune status generated by primary treatment strongly modifies the degree of disease-induced mortality and susceptibility for all strains. Hosts with prior exposure to high-virulence strains (Fig. 2, red lines) had the lowest disease-induced mortality and susceptibility among all primary treatment groups. Thus, by generating stronger host protection during primary infection (Fig. 1), high-virulence strains effectively exclude low-virulence strains from future infections of that host.

We next asked how the observed relationship between virulence and protection against reinfection alters the optimal or evolutionarily stable level of virulence in a pathogen population (Fig. 3). We used a two-strain SIRS (susceptible-infected-recovered-susceptible) model with the

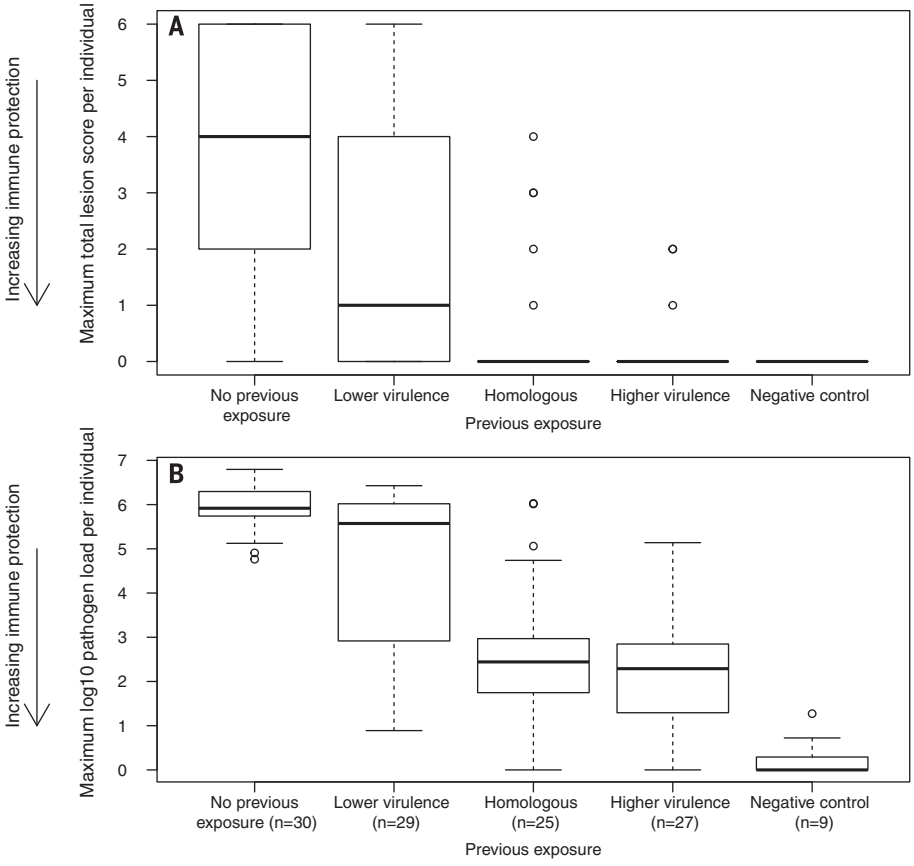

Fig. 1. Lesion scores and pathogen loads for different exposure treatments. Clinical signs of conjunctivitis (**A**), which predict mortality in the wild, and conjunctival pathogen loads (**B**) in hosts with no previous exposure (positive controls) or previous exposure to *M. gallisepticum* strains that either were homologous or had different levels of virulence. Negative controls received sterile medium for both exposures. Host protection was strongest when the primary exposure strain was of higher virulence than the secondary strain, indicating an effect of strain virulence separate from strain homology effects. Box plots show the maximum observed lesion score (on a scale of 0 to 3 per eye, summed within sampling data for a maximum score of 6) or the conjunctival pathogen load for each of 120 individuals from six postexposure measurements. The levels of virulence of the previous-exposure strains are grouped categorically here for clarity (differences among treatment groups were analyzed by the Kruskal-Wallis test; eye score, $H = 68.16$, df = 4, $P < 0.0001$; pathogen load, $H = 76.80$, df = 4, $P < 0.0001$), but virulence was treated as a continuous variable in the model and analysis. *n*, number of individuals.

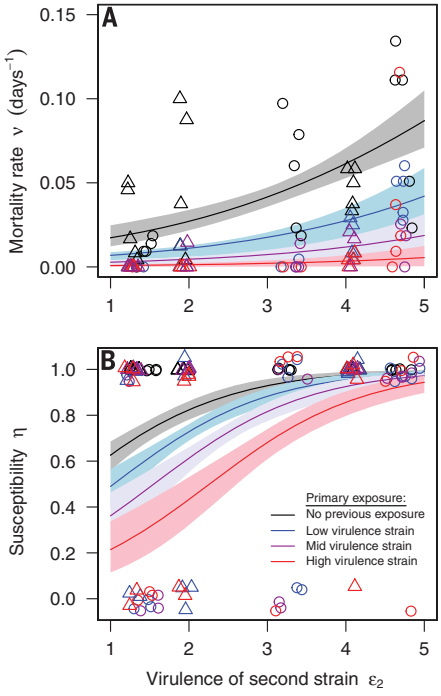

Fig. 2. Model parameters as functions of pathogen virulence, fit to empirical data. Higher virulence of the currently infecting (i.e., secondary) strain is associated with higher host mortality (**A**) and host susceptibility (**B**), but host prior exposure (colored lines) reduces disease-associated mortality and susceptibility. Prior exposure to a high-virulence strain provides the most protection and thus results in the lowest host mortality and susceptibility. The effect of virulence of the primary strain was fit as a continuous function, but to visualize effects, lines and points show three categories of primary strain virulence: low (blue), intermediate (purple), or high (red). Shading around lines denotes bootstrapped 95% confidence intervals incorporating error in virulence estimates. Circles and triangles represent data from experiments one and two, respectively. Mortality rates (A) were inferred to scale linearly with conjunctival lesion scores; susceptibility (B) was fit to binomial infection status (yes or no) inferred from conjunctival pathogen presence. Because distinct strains were used in each experiment (Tables 1 and 2), lines show the function averaged for the two experimental strains in each virulence category (e.g., low virulence). See fig. S4 for functions and data separated by experiment.

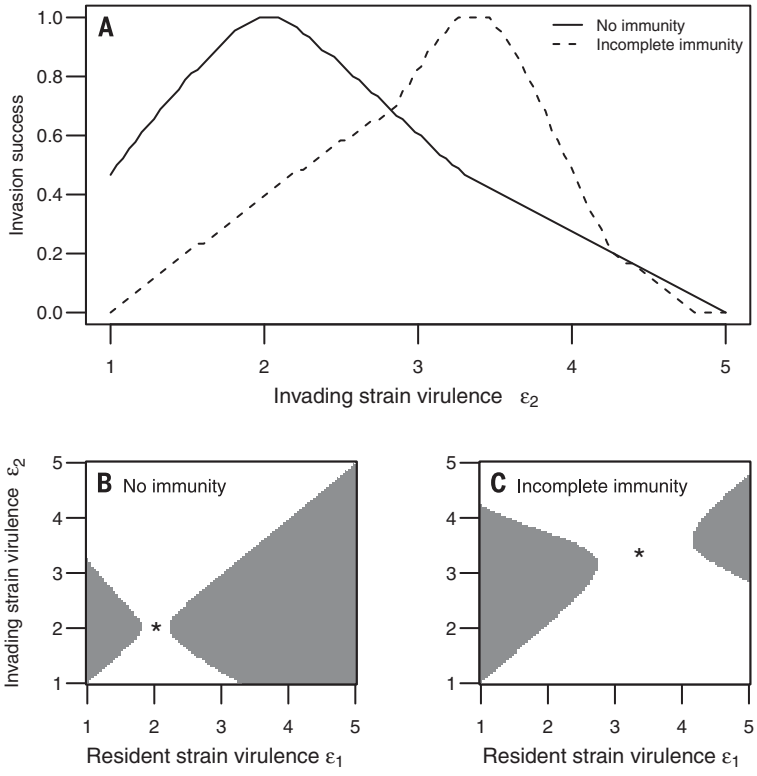

Fig. 3. Optimal virulence in models with no immunity and incomplete immunity. (**A**) The empirically observed effects of incomplete immunity result in an almost twofold increase in optimal virulence relative to that in a model with no immunity. Pathogen fitness is measured as invasion success, computed from the proportion of resident pathogen parameter space over which an invader of a given virulence level was able to successfully displace the resident pathogen in the pairwise invasibility plots (PIPs) for each model (**B** and **C**), scaled to a maximum invasion value of 1 [see supplementary materials and (*35*)]. Shaded areas in the PIPs show parameter space for which a new mutant introduced at very low densities was able to invade a population with equilibrium densities of the resident strain. Nonshaded areas indicate parameter space where the resident strain could not be competitively displaced, and the asterisks in the center of this space thus mark the evolutionarily stable strategy for each model. Susceptibility (η) and mortality (υ) were both continuous functions of strain virulence (ε) (Fig. 2); all other parameters were held constant (equations S2).

Table 2. Relative virulence and log_{10} conjunctival pathogen loads (means ± SEM) for experiment two. The experimental design for experiment two was identical to that for experiment one (Table 1) but used distinct strains. Strains are ordered from low to high virulence (left to right and top to bottom), and relative virulence terms describe the primary exposure strain relative to the secondary exposure strain. As in Table 1, pathogen loads tend to increase top to bottom (with increasing virulence of the secondary strain) but decrease left to right within a row (with increasing virulence of the primary strain). N/A, not applicable.

	Relative virulence and mean pathogen load for primary exposure			
Secondary exposure	**Sterile medium (control)**	**CA2009 low (ε = 1.28 ± 0.34)**	**NC1995 intermediate (ε = 1.95 ± 0.35)**	**VA2013 high (ε = 4.08 ± 0.34)**
CA2009	No prior exposure 2.82 ± 0.33	Homologous 0.68 ± 0.16	More virulent 1.14 ± 0.23	More virulent 1.05 ± 0.20
NC1995	No prior exposure 3.67 ± 0.91	Less virulent 0.86 ± 0.33	Homologous 0.56 ± 0.08	More virulent 0.74 ± 0.14
VA2013	No prior exposure 3.75 ± 0.37	Less virulent 3.00 ± 0.11	Less virulent 2.64 ± 0.23	Homologous 1.51 ± 0.47
Sterile medium (control)	Negative control 0.0 ± 0.0	N/A	N/A	N/A

empirically fit mortality and susceptibility functions (equations S2 and table S3) and compared this incomplete immunity model with one that still allows for higher host mortality and transmission for higher-virulence strains (*15*) but does not include immune protection from prior exposure. We applied an adaptive dynamics approach by simulating the invasion of a new mutant into a population with an endemic resident pathogen strain for a range of virulence levels of both the resident and invading strains (see supplementary materials). In the resulting pairwise invasibility plots (PIPs), the protective effects of host immunity shifted the pathogen's evolutionarily stable strategy to a virulence level almost twice as high as that in a model with no host protection (Fig. 3). This result, which is robust even when additional protective effects of strain homology are added to the model (fig. S3), is driven by two distinct mechanisms favoring greater virulence. First, because disease-induced mortality is a key cost of virulence, higher-virulence strains benefit the most from the reduction in host mortality (Fig. 2A) generated by incomplete immune protection. Second, the stronger protection provided by higher-virulence strains reduces the pool of previously infected hosts available for reinfection by lower-virulence strains (Fig. 2B). We evaluated the general applicability of our two-strain model to other study systems by conducting a numerical and analytical global sensitivity analysis. We found that a stronger relationship between virulence and immune protection enhances the competitive advantage of the more-virulent strain (tables S4 and S5). Thus, incomplete immunity should favor the evolution of greater virulence in any system in which higher-virulence strains generate a stronger protective effect against reinfection than lower-virulence strains (tables S4 and S5).

Our results, combined with high *M. gallisepticum* prevalence (*8*, *16*, *17*) and recovery rates (*6*) in the wild, indicate that host immunity plays a prominent role in the evolution of increased *M. gallisepticum* virulence in nature (*10*). Additional evolutionary processes, such as selection favoring higher transmission rates for more-virulent strains (*15*), may also contribute to the observed increases in virulence. Adaptation to a novel host is unlikely to explain the virulence increases on both coasts, as the more evolutionarily derived California strains of *M. gallisepticum* have lower virulence than eastern strains (Tables 1 and 2) (*10*, *18*). Although evolution of host resistance can lead to increased virulence (*19*) and may play a role in this system (*20*), there was no evidence of evolved host resistance in two studies conducted after the detected increases in virulence (*21*, *22*). Thus, our results suggest that *M. gallisepticum* virulence increased in both eastern and western populations as hosts with incomplete immunity became more common in each population.

The effects of incomplete immunity described here are arguably a specific case of a broader phenomenon whereby increased virulence is favored by quantitative host variation in susceptibility, whether due to host genetic variation (*23*), imperfect vaccines (*5*), or innate immune priming

(*24*). Disentangling differences in susceptibility versus infectiousness is a difficult problem in any system, and we do not attempt to do so here. Our model attributed differences in infection rates to what we term "host susceptibility" (η in equations S2), as infection occurred through inoculation with equal pathogen doses. It is likely that lower pathogen loads in hosts with prior exposure (Fig. 1B) will also lead to lower infectiousness and thus less transmission by those individuals. Although our models allowed for higher transmission rates for more-virulent strains in both scenarios (Fig. 3), we did not vary transmission rates with host prior exposure. Further, although our data support reduced infection length with prior exposure (fig. S2), we were unable to robustly quantify this effect and thus assumed equal infection lengths. Thus, our model is likely conservative, as selection for higher virulence should be even stronger if infectiousness and infection length also vary with host prior exposure.

Previous studies argue for great care in designing vaccines because incomplete protection can select for increased virulence in the targeted pathogens (*1*, *3*, *5*). Our study suggests that pathogens can readily evolve toward higher virulence due simply to the imperfect nature of host immune memory, whether via adaptive or innate responses (*14*). Despite historical focus on the small subset of pathogens that confer complete and lifelong immunity, incomplete immunity following infection is widespread in humans (*25*–*28*) and other animals (*29*–*32*). Because there are likely many systems where more-virulent pathogen strains stimulate stronger immune responses and thus provide stronger protection (*33*, *34*), the fitness advantages to higher virulence demonstrated here are relevant for a range of host and pathogen systems, including humans. Overall, our results show that the same immune systems that evolved to protect hosts from infection can drive the evolution of more-harmful pathogens in nature.

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ACKNOWLEDGMENTS

This work is dedicated to the memory of Paul Williams, who inspired us all with his insights and dedication. We thank L. Kirkpatrick, J. Adelman, S. Moyers, and numerous undergraduates for technical assistance. D. J. Páez and three anonymous reviewers provided helpful comments on the manuscript. This work was funded through NIH grant 5R01GM105245 to D.M.H. under the NIH-NSF-USDA Ecology and Evolution of Infectious Diseases program. Birds were captured under permits from the Virginia Department of Game and Inland Fisheries (044569) and the U.S. Fish and Wildlife Service (MB158404-1). All data and code to understand and assess the conclusions of this research are included in the main text or are available via Dryad Digital Repository (doi:10.5061/dryad.435h5).

SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/359/6379/1030/suppl/DC1
Materials and Methods
Supplementary Text
Figs. S1 to S4
Tables S1 to S5
References (*36*–*49*)

30 June 2017; accepted 12 January 2018
10.1126/science.aao2140

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Science **359** (6379), 1030-1033.
DOI: 10.1126/science.aao2140

Ratcheting up wild virulence

Partially protective vaccination can sometimes select for increasingly virulent pathogens. Fleming-Davies *et al.* asked what happens in a natural system. In the United States, the house finch population is suffering an increasingly virulent epidemic caused by *Mycoplasma gallisepticum*. The pathogen induces incomplete immunity that clears less virulent pathogens and offers partial protection against strains of greater virulence. In the birds, the partial immune response does away with competition from the less virulent pathogens. The partial immunity of the host also hinders replication of the more virulent pathogens enough to allow some birds to survive. This allows increasingly virulent forms of the pathogen to be transmitted.

Science, this issue p. 1030